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# INTRADERMAL IMMUNIZATION OF MONKEYS WITH ONE SET OF INJECTIONS OF POLIOMYELITIS VIRUS

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The problem of artificial immunization in poliomyelitis continues to hold attention because of the reappearance of epidemics of poliomyelitis in different parts of the world and the increasing knowledge of immunity to the class of so-called virus diseases, of which poliomyelitis is one example. That it is possible to produce an artificial active im-

A paper published in the December number of this *Journal* by Michael Heidelberger and Forrest E. Kendall, entitled "A quantitative study of the precipitin reaction between Type III pneumococcus polysaccharide and purified homologous antibody," was awarded an A. Cressy Morrison Prize in December, 1929, by the New York Academy of Sciences. This statement is made in conformity with the conditions under which the prize is given.

strain is used, the result is different:—The monkeys of our test became ataxic, tremulous, and excited about a week after the third intracutaneous injection, or, in other words, presented the appearances manifested by ordinarily inoculated monkeys a day or two before the onset of paralysis. Of the four monkeys showing the preparalytic symptoms, three recovered completely—that is, the symptoms aborted—while the fourth became typically paralyzed and succumbed to experimental poliomyelitis, the spinal cord and medulla revealing characteristic lesions. Flexner and Lewis described the occurrence of isolated instances of paralytic poliomyelitis in monkeys undergoing subclinical immunization, and Aycock and Kagan and Stewart and Rhoads

noted the same occurrence. The greater number of monkeys, when injected repeatedly with weak virus strains, become actively immune without exhibiting abortive symptoms.

These observations raise the question whether repeated minute inoculations of poliomyelitis virus do not act in certain instances to render the treated monkeys, not more resistant but more susceptible to subsequent injections into skin and subcutaneous tissue, such as would usually be ineffective. It seemed possible that a larger quantity of potent virus given intradermally at one injection might accomplish active immunization without inducing any symptoms whatever. The experiment now to be recorded constitutes a test of this second point.

#### EXPERIMENTAL

Four *Macacus rhesus* monkeys were prepared by shaving the ventral surface from the clavicle to the pubis, and cleaning the skin carefully with alcohol. The material used was a 5 per cent physiological saline suspension of recently glycerolated spinal cord of the pooled, mixed virus strain. This strain is one of known constant activity; doses as small as 0.005 cc. of Berkefeld filtrate are uniformly infective when inoculated intracerebrally, and repeated small doses of the same strain given intradermally produce poliomyelitis. Inoculation was performed with a fine needle on a tuberculin syringe, and evenly spaced blebs of the material, about 0.5 cm. in diameter, were introduced into the skin, until the entire amount, 16 cc., had been given. Great care was observed to prevent the material from entering the subcutis, to obviate the possibility of too rapid diffusion of the virus. The animals were examined daily both for symptoms of poliomyelitis and for local reaction in the skin area treated. At no time were any abnormalities observed.

A month was allowed to elapse and the animals were bled for neutralization tests. This procedure was deemed advisable since Stewart and Rhoads (1) have shown that the power of serum to neutralize virus is a more delicate test of immunity than is the resistance of an animal to actual inoculation of the virus itself. Each serum obtained was set up in a volume of 0.99 cc. against 0.01 cc. of a Berkefeld N filtrate, prepared from a 5 per cent physiological saline solution suspension of fresh poliomyelitis spinal cord. The mixtures were placed in the incubator for two hours and in the icebox overnight. The following morning each was inoculated intracerebrally in a normal monkey.

The power of the serum to neutralize virus was compared with the resistance of the animals to actual introduction of infectious material into the central nervous system. The treated monkeys with a normal control were inoculated in the cisterna magna with 0.01 cc. of virus filtrate—about five minimal lethal doses of the same strain. Since intracerebral inoculation involves considerable trauma the cisternal route was selected as providing conditions more nearly resembling those in man when infection occurs (4).

*Results*

The results of the experiments are summarized in the tables. The neutralizing power of the sera tested is shown in Table I. Neutralization was complete in every case, whereas in the control instance an

TABLE I  
*Serum Neutralization*

Monkey No.	Treatment			Symptoms	Neutralization		Result
	Virus	Route	Amount		Serum	Virus	
			cc.		cc.	cc.	
1	M. V.	Intradermal	15	0	0.99	0.01	No symptoms
2	M. V.	Intradermal	15	0	0.99	0.01	No symptoms
3	M. V.	Intradermal	15	0	0.99	0.01	No symptoms
4	M. V.	Intradermal	15	0	0.99	0.01	No symptoms
5	—	Control	—	—	0.99	0.01	Typical poliomyelitis

TABLE II  
*Cistern Test*

Monkey No.	Treatment			Symptoms	Inoculation		Result
	Virus	Route	Amount		Route	Dose	
			cc.			cc.	
1	M. V.	Intradermal	15	0	Died—intercurrent disease		—
2	M. V.	Intradermal	15	0	Cistern	0.01	Typical poliomyelitis 10 days—recovered
3	M. V.	Intradermal	15	0	Cistern	0.01	No symptoms
4	M. V.	Intradermal	15	0	Cistern	0.01	No symptoms
5	—	Control	—	—	Cistern	0.01	Typical poliomyelitis 4 days—died

equal amount of virus treated with normal serum led to typical poliomyelitis after a seven day incubation period. It therefore appears clear that the intradermal inoculation described produced a definite degree of immunity.

Table II shows the results of intracisternal inoculation of virus into

the monkeys that had received the immunizing treatments. As one animal of the series died of intercurrent disease, only three treated animals could be tested. Two remained free of all symptoms, while the third developed typical symptoms of poliomyelitis, became paralyzed in extremities and back, was prostrate for some time, but recovered, although marked residual paralysis persisted. The inoculated control animal, on the other hand, became paralyzed on the fourth day, prostrate on the fifth, and died on the sixth. The difference in the results of the tests in the treated and control animals is rendered more striking by the fact that the strain of virus used, when injected into normal monkeys, was almost invariably fatal within eight days.

#### CONCLUSIONS

1. By means of a single large dose of poliomyelitis virus, distributed at a number of intradermal sites, active immunity has been produced in *Macacus rhesus* monkeys, as shown by skin neutralization and intracisternal tests.

2. In the small series (four animals) of monkeys so treated, neither abortive nor paralytic signs of experimental poliomyelitis appeared.

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# THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY

## VI. THE EFFECT OF ENDOTHELIAL BLOCKADE ON THE STORAGE AND DISTRIBUTION OF NEOARSPHENAMINE

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(Received for publication, September 4, 1929)

For two main reasons scientific workers have studied the fate of arsphenamine and of its various derivatives after parenteral introduction into the body. It is important to know the extent to which the drug is capable of penetrating into the various tissues and the duration of its retention if we are to form any opinion on the degree of its toxicity and its power of reaching parasites in remote places. Of equal importance, though of more theoretical nature, is the knowledge of what kinds of cells are concerned with the storage of the chemotherapeutic agent and what physical and chemical changes the drug undergoes in the body. These questions must be answered before one can hope to throw any light on the mechanism of the chemotherapeutic effect.

A brief review of the more recent literature shows that arsenicals of the arsenobenzene type upon intravenous injection leave the blood stream largely within a few minutes to be taken up subsequently—in decreasing order of amount—by the liver, spleen, lungs, bone marrow, kidneys, brain and skeletal muscles (Voegtlin and Thompson (1), Kolls and Youmans (2), Clausen and Jeans (3), Minot (4), Bulmer (5), Wechselmann, Lockemann and Ulrich (6)). The excretion of the drug with the feces and urine apparently sets in very rapidly (2 to 5 minutes after the injection) and continues for the following 24 to 48 hours, (Beeson and Albrecht (7), Clausen and Jeans, Underhill and Davis (8)) after which time the process of elimination is practically completed. According to several authors traces of arsenic may be detected in the excreta up to much later periods and at the end of 48 hours more than 50% of the injected dose is said to remain quantitatively unaccounted for. As a matter of fact, Voegtlin and Thompson established a definite relation between the velocity of excretion and the chemotherapeutic



efficiency, which indicated to them that "temporary retention of the arsenic is unquestionably an essential requirement for good chemotherapeutic action."

Dale (9) has presented an impressive array of facts which would seem to throw some doubt on the validity of the earlier conception of Ehrlich according to which the chemotherapeutic effect of a given substance is determined by the relation between parasitotropism and organotropism—a strong affinity for the protoplasm of the parasite and a weak affinity for that of the cells of the host being highly essential for good chemotherapeutic action. The difficulties encountered in proving by chemical means a differential affinity for parasite and tissue cells together with other reasons have since induced an increasing number of investigators to regard some participation of the host as indispensable to the accomplishment of the chemotherapeutic effect. More serious objections to the Ehrlich axiom arose from the fact that removal of the spleen or blockade of the reticulo-endothelial system resulted in a marked impairment or, in some cases, complete abolition of the chemotherapeutic action of arsphenamine and other chemotherapeutic agents (Kritschewski and Meersohn (10), Feldt and Schott (11), Jungeblut (12)). The intervention of a specialized system of cells was thus, for the first time, proved to be indispensable for the accomplishment of the full chemotherapeutic effect. More significance was lent to these empirical findings, when v. Jancso (13) and Imenez de Asua and Kuhn (14) succeeded, independently, in demonstrating by special histochemical staining methods the actual presence of the drug in the histiocytes of the liver and in other cells belonging to the reticulo-endothelial system.

In attempting to explain the deleterious effect of blockade or splenectomy on the chemotherapeutic efficiency of arsphenamine, different interpretations were offered. There are of course those who believe that the destruction of the parasite by the drug is due to the combined action of chemical agencies and immune bodies (Kolle and others). Extremists deny any interaction between parasite and drug and attribute the sterilization effect solely to enhanced cellular activity (Feldt). Among other factors we (Jungeblut) discussed particularly the possibility that the transformation of the compound inactive *in vitro* into the highly parasitocidal drug *in vivo* may take place in the reticulo-endothelial cells. It seems to us, that evidence since produced, would not tend to corroborate this hypothesis, at least not in its original simple form, and we are now inclined to maintain a viewpoint, which approaches the opinion of Kritschewski (15), voiced after revision of his first attempt of explanation. We believe that rapid initial storage and gradual subsequent release of arsphenamine is an indispensable prerequisite if the drug is to display an optimum chemotherapeutic effect in the body. It is highly probable that a similar mechanism comes into play with other drugs, such as Bayer 205 (Germanin) and quinine (Sei (16), Roehl (17), Freund (18), Mayer and Zeiss (19), Giemsa (20), and Tchapkewitch (21)). Whether the rôle of the reticulo-endothelial system during this phase is simply one of providing a depot and store for the chemotherapeutic agent, or whether important chemical changes are mediated by those cells is, at present, largely a matter of speculation, at least as far as the

second possibility is concerned. The first question, however, is readily open to direct experimentation.

The present paper deals with an attempt to determine by quantitative chemical analysis the rate of disappearance of neoarsphenamine from the circulation after intravenous injection in blocked and normal animals and to compare the arsenic content of the liver and spleen within the two groups.

#### EXPERIMENTAL WORK

In order to secure more complete information, the work was planned to include observations with three different species of animals, *i.e.* guinea pigs, rabbits, and mice. Guinea pigs and rabbits were used for measurements of arsphenamine in the blood, while determinations of the arsenic content of the liver and spleen were carried out with mice. The method of blockade followed closely the procedure employed in former experiments. Inasmuch as the Abelin reaction (22) has been found reliable by previous workers, we have used this test in quantitative form for the determination of neoarsphenamine in samples of blood. The arsenic determinations in the tissues were carried out with the Gutzeit method, as modified by Sanger and Black (23). The results obtained with the three different series are reported under separate headings as follows:

*Series I.*—A total of sixteen guinea pigs weighing from 300 to 400 grams were used. Eight of these were normal controls, the remaining eight received by intravenous injection 1.5 cc. of a 1:5 dilution of India ink. All animals were injected intravenously with 1 cc. of a 1:70 dilution of neoarsphenamine, the interval between blocking injection and injection of the drug being approximately 24 hours. One half of the animals were bled to death five minutes after the injection of neoarsphenamine, the other half at the end of twenty minutes. The blood was allowed to clot and 1 cc. of clear serum was used from each animal as sample in carrying out the Abelin test. We followed the original method described by Abelin and obtained rather sharp and well defined colour reactions for a range of neoarsphenamine dilutions varying from 1:1000 to 1:50,000. Although the reaction as originally described by Abelin was only a qualitative test, it may be used for quantitative determination with a fair degree of accuracy by comparing the colour shades obtained with the test material with those of a freshly prepared scale of neoarsphenamine solutions of known concentrations. The results obtained in this series appear in Table I.

In spite of individual variations from animal to animal, which undoubtedly depend to a large extent upon the varying total blood supply, the figures show very clearly a much slower absorption of neoarsphenamine from the blood stream of blocked animals as compared

with normal controls. Taking the average as a basis of comparison it would appear that the ratio is approximately 1:2. Assuming the average total volume of plasma of these guinea pigs to be about 7 cc., the figures mean that five minutes after the injection the blocked animal still retains in the blood about one half of the total dose of neoarsphenamine injected while the concentration of the drug in the blood of the normal animal is only one fourth. After the twenty

TABLE I

*Determinations of Neoarsphenamine in the Blood of Blocked and Normal Guinea Pigs\**

(Abelin Test)

Blocked** Guinea Pigs	Interval ***		Normal Guinea Pigs	Interval***	
	5 min.	20 min.		5 min.	20 min.
	<i>mg. of Neo per 1 cc. of serum</i>			<i>mg. of Neo per 1 cc. of serum</i>	
1	1.18		9	.59	
2		.47	10		.39
3	1.3		11	.36	
4		1.0	12		.33
5	.40		13	.40	
6		.31	14		.20
7	1.2		15	.80	
8		.72	16		.58
Average.....	1.02	.63	Average.....	.54	.38

\* All animals received intravenously 1 cc. of a 1:70 dilution of neoarsphenamine. Total dose injected: 14.28 mg. Neo.

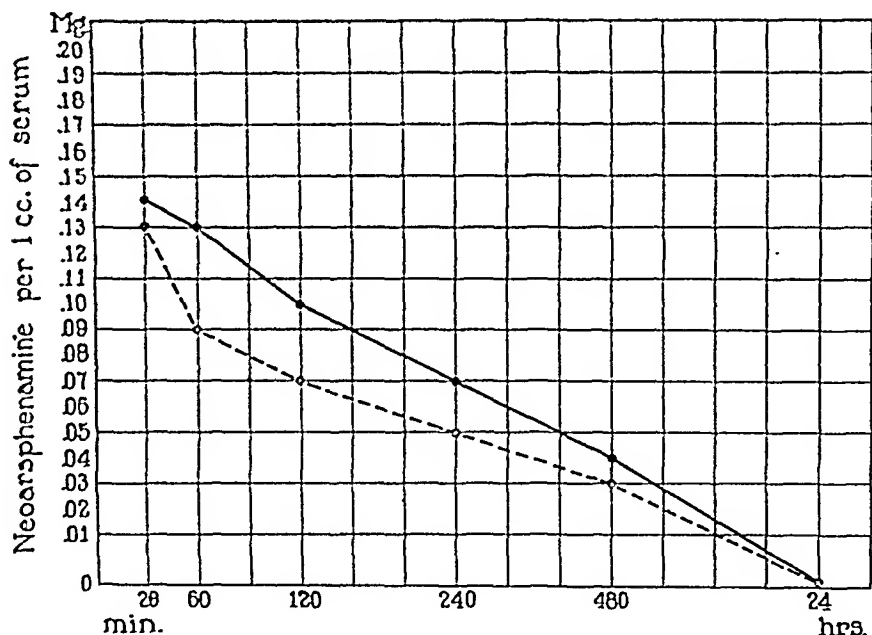
\*\* These animals received intravenously 1.5 cc. of a 1:5 dilution of India ink 24 hours before injection of the drug.

\*\*\* Time elapsed between injection of the drug and obtaining of sample.

minute interval the figures are reduced to approximately one fourth of the total dose for the blocked animal against one eighth for the controls. Those figures are of course not absolute values, the object of the experiment lying chiefly in the relative comparison between experimental and control animals. It will also have to be remembered, that the Abelin reaction is no test for arsenic, but indicates merely the presence of the unchanged drug, *i.e.* the whole organic complex.

In order to supplement the data obtained in the first series with information regarding the rate of absorption during longer periods of time in the same animal, we have followed the disappearance of the drug from the circulation of rabbits in a second series of experiments.

*Series II.*—A total of four rabbits, two of them blocked by a preceding intra-venous injection of India ink (5 cc. of a 1:5 dilution), two serving as normal



GRAPH 1. Elimination of neoarsphenamine from the blood of blocked and normal rabbits. Total dose injected: 30 mg. neoarsphenamine. ——— Blocked animal (combined results of two different tests). - - - - - Normal animal (combined results of two different tests).

controls, received each 3 cc. of a 1:100 dilution of neoarsphenamine into the ear vein. Blood samples (5 cc.) were obtained by heart puncture from each rabbit at various intervals, covering periods of from twenty minutes up to 24 hours since the injection of the drug. The Abelin test was carried out with 1 cc. of clear serum from each sample as described before.

Graph 1 shows the different curves of elimination. Although the differences between blocked animals and controls are not as marked as in series I, they show a distinctly slower elimination of the drug

from the circulation of the blocked rabbit. Our observations in normal guinea pigs and rabbits show otherwise a somewhat more abrupt fall in concentration of the circulating arsenical than the figures, which Schreus and Hollaender (24) established, would indicate for humans.

TABLE II

*Determinations of Arsenic in the Livers of Blocked and Normal Mice Following the Injection of Neoarsphenamine\**

(Gutzit Method)

Blocked Mice**				Normal Mice		
Interval	Weight of liver	Neo*** per 0.5 g. of tissue	Neo*** per whole organ	Weight of liver	Neo*** per 0.5 g. of tissue	Neo*** per whole organ
minutes	g.	mg.	mg.	g.	mg.	mg.
5	1.80	.23	.828	1.20	.33	.792
"	1.13	.15	.339	1.02	.50	1.02
20	1.15	.21	.483	1.07	.43	.920
"	1.37	.25	.685	0.72	.60	.864
30	3.15	.20	1.26	1.50	.50	1.50
"	0.90	.10	.18	1.60	.30	.96
60	1.12	.25	.56	1.49	.40	1.192
"	1.38	.10	.276	1.25	.30	.75
120	1.14	trace	—	1.12	.05	.112
"	1.00	.05	.10	1.40	trace	—

\* All animals received i.v. 1 cc. of a 1:250 dilution of neoarsphenamine. Total dose injected: 4 mg. Neo.

\*\* These animals received i.v. 1 cc. of a 1:15 dilution of India ink 24 hours before injection of the drug.

\*\*\* The neoarsphenamine figures are computed by multiplying the arsenic values by five. The brand of neoarsphenamine used contained about 20% As.

In a third series of experiments the arsenic content of the liver of blocked and normal mice was determined, following the intravenous injection of neoarsphenamine.

*Series III.*—A total of 20 animals, which weighed from 20 to 25 grams, received a uniform dose of 1 cc. of a 1:250 dilution of the drug. One half of them were

normal controls, the other half had been blocked by a preceding intravenous injection of India ink (1 cc. of a 1:15 dilution). Duplicate animals in either group were killed at five, twenty, thirty, sixty, and 120 minutes, respectively, after injection of the drug, and the liver, including the gallbladder, was carefully removed *in toto*. After accurately determining the weight of the organ, an aliquot portion of the organ (0.5 g.) was finely ground in the mortar. The organic matter in the tissue being destroyed by the usual treatment with boiling  $\text{H}_2\text{SO}_4$  and  $\text{HNO}_3$ , the arsenic content in the residue was determined by Sanger and Black's modification of the Gutzeit method. The computed neoarsphenamine figures are tabulated in Table II.

As may be gleaned from Table II the figures show on an average approximately half as much arsenic in the blocked liver as in the normal organ, comparing at similar intervals gram for gram of tissue. Limiting our observations to the first hour we found in the whole normal liver approximately from one fourth to one fifth of the total amount of arsenic injected while the fraction of arsenic present in the whole blocked liver has varied from one tenth or less to one fifth of the total dose. Although the number of determinations is small and the individual figures show considerable variation, it would seem that the maximum concentration of the drug in the liver is established during the course of the first hour, after which time there is a rather rapid decline in the amounts of arsenic recovered. Several attempts to measure the arsenic content in the spleen have yielded amounts too small to be measured accurately with our technique. Our results have thus failed to bear out Schlossberger's (25) expectations.

#### DISCUSSION

Our findings are in the main in agreement with the experience of many authors that blockade of the reticulo-endothelial system leads to a slower elimination of "storable" colloids from the blood stream and consequently to a less complete impregnation of the reticulo-endothelial cells with these substances (for a review of the literature see Jungeblut (26)). As far as the present work is concerned it appears that the absorption of neoarsphenamine from the blood stream after intravenous injection of the drug is materially retarded in guinea pigs which received a single blocking injection of India ink 24 hours before, as compared with normal controls. This difference is more apparent at the earlier intervals following the injection of the drug

than at later stages. The elimination curves in the rabbit show a less marked, but nevertheless very regular and characteristic lag of the blocked animal against the normal control. As far as the final residue in the circulation after 24 hours is concerned, we have not been able to detect any differences with either group of animals.

The above results harmonize well with the information obtained from arsenic determinations in the tissues of blocked and normal mice after the intravenous introduction of the same drug. The facts here indicate in brief that the concentration of neoarsphenamine in the blocked liver is less than one half of the amount present in the normal organ, comparing at similar intervals during the first hour's observation gram for gram of tissue. Blockade of the reticulo-endothelial system, in other words, alters profoundly the storage and distribution of neoarsphenamine, causing shortly after injection of the drug more neoarsphenamine to remain in the circulation and less to accumulate in the tissues.

To what extent the observations made in this paper serve to elucidate the mechanism of chemotherapeutic action, is a question which at present can be answered only tentatively. Those who argue that the arsphenamine in the tissues is negligible for the chemotherapeutic action and accordingly credit the circulating arsphenamine with the sterilization effect (v. Jancso, Schlossberger, Wechselmann, Lockemann and Ulrich) will find it difficult to reconcile such an hypothesis with the fact that blocked animals, whose blood actually contains larger amounts of the drug, die from the experimental infection while normal controls survive with much smaller concentrations of arsphenamine in the circulation. On the other hand, if we are to attribute causal significance to the process of storage by the tissue cells for the chemotherapeutic efficiency of the drug, we are baffled by the recent paradox observations of v. Jancso (27). While, according to this author, among the arsenicals of the arsenobenzene type, those which possess a high degree of tissue affinity, also exhibit the highest trypanocidal action, there is no parallelism demonstrable between reticulo-endotheliotropism and the chemotherapeutic efficiency as regards the action of the various derivatives on spirochaete infections. Finally, the peculiar intervention of the intact reticulo-endothelial system is apparently not restricted to the arsenicals, but governs as

well the pharmacodynamics of such totally different substances as Bayer 205 (Germanin).

While evidently the problem is still far from a perfect solution, we feel inclined to attribute the impairment of the chemotherapeutic efficiency of the arsphenamines by blockade of the reticulo-endothelial system in general to an interference with the storage of the drug by the histiocytic cells. Following the indicated trend of thought it would seem that the blocked animal is distinctly handicapped in its fight against the experimentally produced infections by the initial lag of the resorptive power of the liver and possibly by a disturbance of the regulative function of the organ for the subsequent release of the drug, probably in some modified form. The composite picture of the fate of the drug in the blocked animal is necessarily incomplete without knowing the effect of blockade on the rate of excretion through the feces and urine; we therefore prefer to reserve final judgment until such information is available. Further work will also have to show whether *increased* activity of the reticulo-endothelial cells, induced by suitable stimulation, might not result in a *higher chemotherapeutic effect*, an expectation which would not seem to be altogether ungrounded in view of the fact that quite recently Roskin and Romanowa (28) have been able to show that irradiation of the experimental animal with ultraviolet light will enhance the chemotherapeutic effect.

#### CONCLUSIONS

1. Blockade of the reticulo-endothelial system by means of a single injection of India ink caused a marked retention of neoarsphenamine in the blood of guinea pigs during the first twenty minutes of observation after intravenous injection, as contrasted with the rapid disappearance of the drug from the blood of normal controls.

2. Rabbits blocked by a single dose of India ink showed a slower elimination of the drug from the circulation following the first few hours after intravenous injection than corresponding controls.

3. The arsenic content of the liver of mice, which received neoarsphenamine intravenously after a preceding blocking injection with India ink, was appreciably lower than the arsenic content of the normal organ under similar experimental conditions.



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# THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY

## VII. THE EFFECT OF LOCAL AND SYSTEMIC BLOCKADE ON THE TOXIC ACTION OF HETEROPHILE (FORSSMAN) SERA IN GUINEA PIGS

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### INTRODUCTION

During the past few years the relations between anaphylactic phenomena and the function of the reticulo-endothelial system have received a large share of scientific interest. In a critical review we (Jungeblut (1)) have recently surveyed the vast amount of information which has accumulated from work in this field. While all efforts, undertaken with the view point of employing the blockade method in the study of anaphylaxis for the prevention of systemic anaphylactic shock, have yielded but uncertain or contradictory results, more significant observations have been made by Klinge (2) who succeeded, in preventing completely the development of the local Arthus phenomenon in sensitized rabbits, provided the antigen was reintroduced into a tissue area, which had been locally blocked by a suitable dose of trypan blue. The toxic action of heterophile antisera in animals belonging to the 'guinea pig type,' which bears a striking resemblance to the symptomatology and pathology of allergic reactions, is characterized by the occurrence of severe tissue lesions, more extensive than what is generally observed in typical protein hypersensitiveness. It has therefore seemed worthwhile to us to investigate, to what extent cells of the reticulo-endothelial system are involved in the mechanism of this reaction.

As far as we are aware, there is only one observation of Forssman and Skoog (3) recorded in the literature, which deals with the present subject. These authors have reported, that injections of India ink,

while protecting against the so-called 'carotal symptom complex,' have no influence on the occurrence of the inverse anaphylactic reaction.

### EXPERIMENTAL WORK

In the following experiments we studied the effect of systemic blockade upon the development of systemic shock in the guinea pig following the injection of Forssman sera. At the same time we investigated the influence of local blockade on the occurrence of the local skin reaction in the animal.

For the first part of the experiments both blocking agent and heterophile serum were introduced by the intravenous route, while in the second part the serum was injected intracutaneously into areas of skin, which had previously been infiltrated with the blocking substance. We have used India ink as well as trypan blue for purpose of blockade. The work was planned so as to include observations after different intervals between the establishment of blockade and the injection of the toxic serum. The latter was obtained by immunization of rabbits with an emulsion of guinea pig kidney cells; for part of the work we have also employed an anti-sheep cell hemolytic serum.

*Series I.*—In the first series a group of six guinea pigs were injected intravenously with 2 cc. of a 1:5 dilution of India ink and received at various intervals later a dose of 1 cc. of an anti-sheep cell rabbit serum by the same route. Three control animals were injected with the serum alone. This particular serum, upon careful preliminary titration for toxicity, had yielded a minimum lethal dose of 0.75 cc.\* The results are tabulated in Table I.

*Series II.*—We have compared in this series the respective effect of India ink and trypan blue as blocking agents under otherwise similar experimental conditions. A total of seven guinea pigs were blocked with 2 cc. of a 1:5 dilution of India ink and an equal number of animals were prepared with 2 cc. of a 1% solution of trypan blue. All fourteen animals were injected intravenously at various intervals since establishment of blockade with 0.75 cc. of an anti-guinea pig kidney cell rabbit serum, the minimum lethal dose of which had been found before to be 0.5 cc. For purpose of control we have included in this series again three normal animals which received only the serum. Table II shows the results obtained.

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\* We have designated as minimum lethal dose the smallest amount of serum, which upon intravenous injection into a guinea pig (weight from 250–350 g.) causes the death of the animal within five minutes. Somewhat smaller amounts have generally killed the animal within from ten to thirty minutes or on the following day.

A study of the results obtained with the two experimental series reveals that blockade of the reticulo-endothelial system has in the majority of the cases resulted in a prolongation of the time of survival of the guinea pigs, without however protecting the animals definitely against the lethal action of the toxic serum. This modification of shock was only fully apparent when several minutes had elapsed between establishment of blockade and the injection of the heterophile serum. As a rule it was noticeable up to a 24 hour interval. We have not

TABLE I

*Effect of Systemic Blockade with India Ink on the Systemic Shock Following Intravenous Injection of Heterophile Serum† in the Guinea Pig*

Guinea pig No.	Injections of India ink	Injection of serum	Interval min.	Results*
1	2 cc. of 1:5 dilution i.v.	1 cc. i.v.	5	Died 3 min.
2	"	"	30	Died 17 min.
3	"	"	60	Died 20 min.
4	"	"	90	Died 26 min.
5	"	"	120	Died 1½ hours
6	"	"	24 hours	Died 2 min.
7	—	"	—	Died 3 min.
8	—	"	—	Died 4 min.
9	—	"	—	Died 3 min.

† Anti-sheep red cells rabbit serum.

\* The symptoms before death resembled very closely those of typical anaphylactic shock. The autopsy findings showed inflation of the lungs and multiple hemorrhages in the kidneys, liver, spleen and lungs.

found either of the two blocking agents—India ink or trypan blue—superior to the other. We infer from the fact that the simultaneous injection of the blocking agent and the immune serum was followed by typical shock, that neither the ink nor the dye has exerted any direct detoxifying action on the serum.

In view of the uncertain results obtained with systemic blockade applied to the study of systemic shock, we have carried out further experiments in which the influence of local blockade on the development of the local tissue reaction was studied.

*Series III.*—In these experiments we have infiltrated a local area of skin (shaved abdominal region) with suitable doses of either India ink (1 cc. of a 1:20 dilution) or trypan blue (0.5 cc. to 1 cc. of a 1% solution). The intracutaneous injection of the serum was carried out at various intervals later; the serum was injected not only into the maximally blocked area but also into the adjoining areas of partially blocked skin and likewise into normal skin areas of the same animal, into

TABLE II

*Effect of Systemic Blockade with Either India Ink or Trypan Blue on the Systemic Shock Following Intravenous Injection of Heterophile Immune Serum† in the Guinea Pig*

Guinea pig No.	Blocking injections	Injection of serum	Interval min.	Results*
1	<i>i.v.</i> India ink. 2 cc. 1:5 dil.	<i>i.v.</i> 0.75 cc.	none	Died 2 min.
2	"	"	10	Died 2 min.
3	"	"	30	Died 4 min.
4	"	"	60	Died 10 min.
5	"	"	120	Died 8 min.
6	"	"	180	Died 12 min.
7	"	"	24 hours	Died 11 min.
8	Trypan blue 2 cc. 1% sol.	"	none	Died 1½ min.
9	"	"	10	Died 2 min.
10	"	"	30	Died 5 min.
11	"	"	60	Died 10 min.
12	"	"	120	Died 9 min.
13	"	"	180	Lived
14	"	"	24 hours	Died 14 min.
15	—	"	—	Died 2 min.
16	—	"	—	Died 5 min.
17	—	"	—	Died 3 min.

† Anti-guinea pig kidney cells rabbit serum.

\* Symptoms and autopsy findings were as described in Table I.

which the blocking agent had not diffused. The latter served as controls. The serum used for these experiments was the same Forssman serum employed in the second series. A minimum amount of .2 cc. of a 1:5 dilution upon titration on the normal guinea pig skin caused a severe local reaction, consisting of hemorrhagic inflammation on the first day to be followed by distinct necrosis on the second or third day. We have employed this dose as test dose in our work.

A total of six guinea pigs were locally blocked with trypan blue as indicated and another six animals were prepared in a similar manner with India ink. The serum was injected immediately, and then at intervals ranging from ten minutes to 24 hours. The tissue reactions following the intracutaneous injections of the Forssman serum were read two hours after completion of the injection and observations were kept up for the next three days. The results, based on the 48 hour reading, appear in Table III.

TABLE III

*Effect of Local Blockade on the Development of the Local Skin Reaction Following Intracutaneous Injection of Heterophile Serum\**

Local blockade with trypan blue (1 cc. of a 1% solution subcut.)					Local blockade with India ink (1 cc. of a 1:20 dil. subcut.)				
Guinea pig No.	Interval min.	Reactions in			Guinea pig No.	Interval min.	Reactions in		
		maxi- mally blocked area	partially blocked area	normal control area			maxi- mally blocked area	partially blocked area	normal control area
1	none	3+	2+	3+	7	none	3+	3+	3+
2	10	—	2+	3+	8	10	3+	3+	3+
3	30	±	2+	3+	9	30	3+	2+	3+
4	60	—	1+	3+	10	60	3+	3+	3+
5	180	+	2+	3+	11	180	2+	3+	3+
6	24 hours	±	±	4+	12	24 hours	3+	2+	3+

\* An anti-guinea pig kidney cell serum was used (.2 cc. of 1:5 dil.). The results are based on a 48 hour reading.

Nomenclature: 4+ = very severe reaction with extensive necrosis,  $1 \times 1$  cm.

3+ = severe reaction with necrosis,  $\frac{1}{2} \times \frac{1}{2}$  cm.

2+ = moderate reaction with slight necrosis,  $\frac{1}{2} \times \frac{1}{2}$  cm.

1+ = slight reaction, no necrosis,  $\frac{1}{2} \times \frac{1}{2}$  cm.

± = very slight reaction, no necrosis, less than  $\frac{1}{2} \times \frac{1}{2}$  cm.

— = no reaction.

As can be seen from Table III, there were either none or at the most extremely faint reactions in the trypan blue-blocked areas, during an interval up to 24 hours after establishment of the blockade. The adjoining areas of partially blocked skin likewise did not respond as much to the toxic action of the serum as the control sites in the normal skin which showed fully developed reactions. On the other hand, the areas blocked with India ink showed lesions fully as severe as the

control sites. The different effect of the two blocking agents, yielding opposite results, was as striking in the positive as in the negative case.\*

### DISCUSSION

The fact that it is not possible by systemic blockade with either India ink or trypan blue to prevent the occurrence of fatal systemic shock following intravenous injection of heterophile immune serum in the guinea pig, is not surprising if we keep in mind the peculiar localization of the heterophile antigen *in vivo*. Forssman (4) considers the kidney as the chief source of the antigen, while according to the same author the liver and spleen contain much smaller amounts. Since the elements of the reticulo-endothelial system are primarily found in the liver and spleen and are absent in the kidney, it would seem that the vital dye has blocked precisely those cells which do not contain Forssman's antigen, leaving free access for the serum to the store of antigen in the kidneys.

In the case of the complete inhibition of the local skin reaction following intracutaneous injection of the heterophile immune serum into areas of skin, locally blocked with trypan blue, it is difficult to reason along analogous lines. Very little is known about the quantities of heterophile antigen present in the skin and subcutaneous tissue, although the severe reactions induced by the serum in the normal skin would, *a priori*, indicate a rich content of antigen in that structure. Details of distribution of the antigen amongst the different types of cells in the skin and subcutaneous tissue have, to our knowledge, never been investigated (5). Since the dye is taken up selectively by the clasmotocytes (histiocytes), and since trypan blue-blocked areas are insusceptible to the toxic action of the serum, it may not be inappropriate to suspect that those cells harbour the antigen. However, it is naturally impossible to make any definite statements on that point without careful histological examination of the particular areas. All the experiment proves is the fact that local blockade has interfered in

\* We have also carried out a few experiments in which either the mouse or the dog was used as the test animal. While the mouse proved insusceptible to large doses of heterophile serum by either the intravenous or intracutaneous route, inhibition of the local skin reaction by local blockade in the dog has not been very clear cut. Our observations are too few, however, to warrant detailed discussion.

some way with the contact between the heterophile antibody in the immune serum and the corresponding antigen in the tissues. As regards the difference between the effect of trypan blue and India ink—the latter being incapable of preventing the full development of the local lesion—we are unable to offer any satisfactory explanation. It is interesting to note, that Klinge has also been unsuccessful in preventing the Arthus phenomenon in sensitized rabbits, when he used India ink as blocking agent, although the experimental conditions are not directly comparable. There is a possibility that the much greater diffusibility of the dye and perhaps its more profound injury to the cell protoplasm may endow it with its peculiar virtues.

#### CONCLUSIONS

1. Guinea pigs blocked by intravenous injection of either India ink or trypan blue succumbed with fatal shock after the intravenous injection of heterophile immune serum, although the time of death was somewhat delayed as compared with normal controls.

2. Local blockade of the skin of guinea pigs with trypan blue inhibited completely the development of the characteristic skin reaction following intracutaneous injection of Forssman serum within the blocked area.

3. Infiltration of the skin of guinea pigs with India ink afforded no protection against the full development of the toxic skin reaction.

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# THE EFFECT OF THE X-RAY ON THE NODULES OF VERRUGA PERUANA

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## PLATE 1

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In a recent paper from this laboratory<sup>1</sup> it was shown that convalescent serum, taken from monkeys which had recovered from experimental verruga, has two evident effects on the disease as produced in monkeys by the inoculation of cultures of *Bartonella bacilliformis*.

The convalescent serum inhibits the development of verrugous nodules, that is, delays the appearance or slows-up the growth, but it does not prevent their ultimate appearance and their reaching considerable size. On the other hand, it effectively prevents the proliferation and persistence of *Bartonella bacilliformis* in the blood stream. It is hoped that the treatment of cases of Carrion's disease in man may be made more effective through the employment of the blood serum of convalescents from verruga and from Carrion's disease itself.

The curative influence of the X-ray on various skin lesions in man has suggested experiments to ascertain whether the rays, applied therapeutically, would affect the evolution of verruga nodules in monkeys receiving skin inoculations of *Bartonella bacilliformis*. The pathology of the lesions indicated that this was probable. In histological structure the verruga nodule<sup>2,3,4</sup> is a circumscribed collection of proliferating endothelial cells, arranged in groups and masses along blood and lymph spaces, in a manner resembling an endothelioma. Ordinary inflammatory changes such as exudation of polynuclears and round cells occur in mild degree only. Furthermore, the verruga nodules in spite of their definitely proliferative nature tend to regression ultimately. In this they differ essentially, of course, from the endotheliomata. The time period during which evolution and regression take place can be gauged quite accurately.

With these facts in mind, we undertook two sets of experiments.

In one we wished to observe the effect of a single application of X-ray on the nodules after they had just started to develop in monkeys, and in the other, to see whether the skin of a normal monkey which had received one application of X-ray would fail to develop verruga nodules on later inoculation.

### *Method*

After the monkey had been shaved it was stretched on a board and the chest and abdomen were covered with a lead-rubber mat 1/16 inch thick. The mat was so placed that the nodule to be treated was in the center of a hole in it 3 cm. square. It follows that an area of 9 square cm. in all was exposed to the X-ray, having at its center the nodule to be treated.

An attempt was made to find and use an X-ray dosage sufficient to produce only an erythema of the skin. Four, six and eight minute exposures at a distance of six inches, with a three inch spark gap and 10 M.A. were tested. It was found that sometimes a six or an eight minute exposure produced desquamation and a serous discharge from the area X-rayed, but in the type experiments reported here only erythema occurred.

### *Treatment of Growing Nodules*

*Macacus rhesus* X-1 was intradermally inoculated with living cultures of *Bartonella bacilliformis*, strain P5, in four places, two on the left side and two on the right side of the abdomen. An inoculation was also made between them into a scarified area on the midline. On the sixth day, nodules 3 to 4 mm. in diameter were distinctly visible and palpable at all the sites of intradermal inoculation (Fig. 1). The following day the two nodules on the left side were treated with X-ray, the left upper nodules for six minutes and the left lower nodule for only four minutes. The nodules on the right side and the scarified area were not exposed to the X-ray, serving as controls. These nodules on the right side progressed steadily; 26 days after inoculation the upper one was 12 mm. and the lower 20 mm. in diameter (Fig. 2). Seventy days after inoculation they had completely regressed and only small scars remained. The nodules on the left side, however, during this period, when those on the right were flourishing, remained stationary in size for about 14 days, and on the 46th day after inoculation had entirely disappeared.

*Macacus rhesus* X-2 was injected intradermally in six places on the abdominal wall, 3 on each side, with cultures of *Bartonella bacilliformis*, strain 2. A scarified area on the midline was also inoculated. Ten days later each intradermal site of inoculation showed a nodule 4 mm. in diameter (Fig. 3). The next day the nodules on the left side were treated with X-ray, 8 minutes for the upper nodule, 6 minutes for the middle nodule and 4 minutes for the lower nodule. The nodules on the right side and the scarified area were not treated and served as controls. On this,

the untreated side, the nodules were 15 mm. in diameter 34 days after inoculation (Fig. 4), and had completely regressed 63 days after inoculation. The nodules on the left, the treated side, remained stationary for about 10 days after treatment, with the surrounding skin slightly hyperemic and edematous. They then gradually regressed and disappeared (Fig. 4).

### *Treatment of the Skin Prior to Inoculation*

In *Macacus rhesus* X-5 the shaven skin of the anterior surface on the left side was exposed to the X-ray for 6 minutes, while the right side remained untreated and served as control. Then, 4 days later, two intradermal inoculations of cultures of *Bartonella bacilliformis*, strain 4, were made on each side of the animal; i.e., two on the treated and two on the untreated side. The nodules on the right, the untreated side, showed the usual progress. On the 46th day after inoculation they were two large pedunculated masses, each 20 mm. in diameter (Fig. 6), and 42 days later they had entirely disappeared. On the left, or treated side, there was marked retardation of the development of the nodules. Eight days after inoculation, they were just palpable and much smaller than those on the right which were red, prominent and 5 mm. across. On the 23rd day the nodules on the right were 10 mm. and 5 mm. respectively, and those on the left had entirely disappeared.

### SUMMARY AND CONCLUSIONS

The supposition that X-ray would affect the developing and the developed verruga nodules experimentally induced in the monkey, has proved correct. The experiments show that the early verruga nodules when exposed to a single, properly graduated dose of X-ray producing merely erythema of the skin, are inhibited in their evolution. Moreover, the skin of *Macacus rhesus* monkeys is modified by a single erythema dose of X-ray in such a way that infection of it with *Bartonella bacilliformis* is rendered more difficult.

These results are sufficiently striking to warrant the trial of X-ray in suitably guarded doses in the treatment of verruga nodules in man. Should the employment of convalescent serum influence the course of Carrion's disease favorably and the use of X-ray bring about a more certain and rapid devolution of verruga nodules in man, two practically applicable therapeutic measures will have been provided for the treatment of the two forms of the human disease.

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## EXPLANATION OF PLATE 1

FIG. 1. *Macacus rhesus* X-1, showing condition of verruga nodules 7 days after inoculation, before treatment with X-ray.

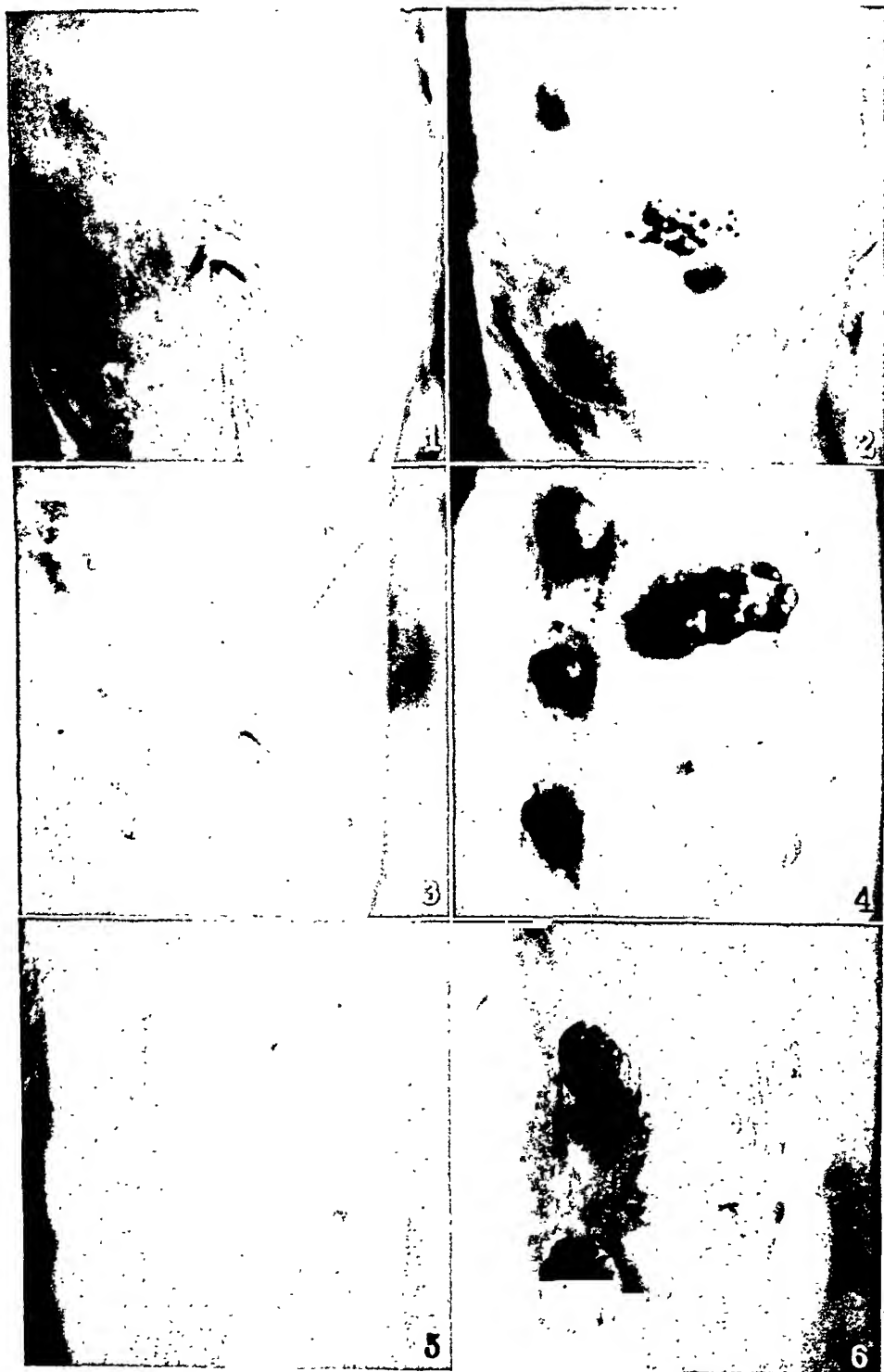
FIG. 2. Same animal 21 days after X-ray treatment. The two nodules on the left side are considerably smaller than the two untreated nodules on the right. Scarified area in the center is untreated.

FIG. 3. *Macacus rhesus* X-2. The six nodules 11 days after inoculation and before X-ray treatment.

FIG. 4. The same animal 23 days after X-ray treatment. The three nodules on the right side of the animal and the scarified area are strongly positive. The three treated nodules on the left side are very much smaller.

FIG. 5. *Macacus rhesus* X-5. The skin of the left side was X-rayed 4 days before inoculation. Picture taken 10 days after inoculation, shows nodules slightly smaller on left side than on right.

FIG. 6. Same animal, 47 days after inoculation, with two large masses on the right side (untreated), but only two small nodules on the left side (treated).



(Muller and Tyler: Effect of X-ray on verruga peruana nodules)



# IS THE LOCAL VASODILATATION AFTER DIFFERENT TISSUE INJURIES REFERABLE TO A SINGLE CAUSE?

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PLATES 2 AND 3

(Received for publication, October 23, 1929)

In recent years physiologists have realized that the capillaries are capable of independent functionings. With the new knowledge have come no few hypotheses concerning the causes for capillary behavior. Important amongst these because of the fruitful experimentation and thought to which it has led is the conception that local vasodilatation in response to injury occurs through the influence of a substance derived from the damaged tissue, this substance being always the same, no matter what the kind of injury. Recently we have encountered facts which render difficult an acceptance of the hypothesis as outlined, while deserving report furthermore for themselves in this present observational stage of inquiry.

The hypothesis of a single underlying cause for all local vasodilatations was foreshadowed by the observations of Ebbecke and others, but finds its most extensive expression in the recent book of Lewis (1). Lewis has described and subjected to an enlightening analysis the "triple response" which develops after various types of skin injury. He concludes that no matter how this response is elicited it is always due to the same chemical agency (the "H substance"). "We have to deal in all instances in which an H substance is under consideration, with a single substance having an invariable chemical constitution;" and the H substance is under consideration, not merely in cases of local injury but in systemic shock, including the anaphylactic (2). Histamine elicits the triple reaction and the evidence "seems sufficient to establish the conclusion that injury of the skin liberates a substance exerting a local action indistinguishable from that of histamine upon the cutaneous nerves and vessels." So "it is difficult to refrain from stating without reserve the simple conclusion that the vasodilator substance considered and the H substance are one and the same, and that this substance is histamine, free or held in loose combination," though a reservation in the matter is maintained for the moment. The assumption that histamine is the effective cause of local pathological vasodilatation induced in many different ways, and of shock



as well, has been the stimulus to much work by others upon the amount of the substance derivable from various organs.

We shall describe experiments which prove that the local vasodilatation consequent upon mechanical injury to the skin differs from that due to histamine. In brief it has been found that the impulse to vascular contraction arising in skin from which the circulation has been cut off prevails over that responsible for local vasodilatation after mechanical injury, with result that the bloodless blanchings known as Bier's spots develop in regions where previously this vasodilatation had reigned. On the other hand the least quantity of histamine which is capable of causing local vasodilatation renders the vessels involved refractory to the contractile influence.

### *The Scratch Tests*

Volunteers were selected who yielded pronounced Bier's spots on the arm under the ordinary circumstances of venous congestion followed by arterial occlusion. The limb was propped horizontally by means of supports located beneath the elbow and wrist,—a procedure which localizes the spots to the upper side (3),—and some time prior to any development of the spots a series of scratches were made with a sterile needle after the skin had been lightly swabbed with alcohol and ether and allowed to dry. Usually two parallel scratches, from 0.6 cm. to 1.0 mm. long and  $1\frac{1}{2}$  to 2 mm. apart were made at intervals of 3 to 4 cm. (Figs. 1-6). Most of these were just deep enough to draw blood in some part of their length. Sometimes the skin was oiled and the progress of the vascular changes was followed with a lens or the binocular microscope.

In a first series of tests the circulation of the arm was interfered with only after the lapse of some minutes, when the characteristic responses to injury had asserted themselves. The findings were so uniform that a single protocol will suffice.

*Test 1.*—The subject, H., was a young man. The right arm, large, quite plump, with a fair, thin skin showing numerous freckles, was propped above a table, the forearm horizontal in partial supination. At five locations between elbow and wrist two scratches were made on the upper side of the arm. Those close to the elbow were in the skin that was highest, as the arm lay, the others being slantingly below this level, in proportion to their nearness to the wrist (Fig. 1). The pairs were laid down in the direction from elbow to wrist during the course of 6 minutes. Eighteen minutes later there was a well-marked, deep pink wheal,  $\frac{1}{2}$  cm. in transverse diameter, underlying each set of scratches, with a pink flare of active hyperemia round about and a pale zone outside (Fig. 1). Two minutes later the

pressure was raised to 70 mm. in a cuff on the upper arm, and after a further 7 minutes to 170 mm. As engorgement proceeded the vascular abnormalities in the neighborhood of the injuries became greatly accentuated (Fig. 2). The pale areola, in special, extended much further than it had previously. The skin under and about the scratches continued to be pink long after that elsewhere had turned violet. Bier's spots began to appear in the skin that was highest within 2½ minutes after the arterial occlusion; and they were especially abundant and pronounced in the regions of response to the scratching. After 12½ minutes of stasis the numerous small blanchings round about the uppermost scratches had largely coalesced and the whiteness had extended up to the scratches and even between these latter (Fig. 3). The spotting was as marked where the skin around the scratches had been red prior to the arterial occlusion as where it had been unnaturally pale (Fig. 3). To obtain Fig. 3 and the subsequent pictures the position of the camera was shifted more nearly over the lesions.

After 23½ minutes of arterial occlusion (30½ after the pressure was raised to 70 mm.) there was a complete blanching almost everywhere in the region where the two highest sets of scratches had been placed, and a less degree of it about those situated lower on the side of the arm. This blanching was complete in the sense that all blood had been driven out of the vessels. The whiteness had intruded itself between the individual scratches of the three uppermost sets and had advanced to the scratches themselves, with result that the latter stood out as dark lines on a white ground (Fig. 4).

The obstructing cuff was now released. A few of the Bier's spots unassociated with the scratches persisted for some seconds but they were soon lost, like the rest, in an active hyperemia which obliterated all previous vascular differences. Only the scratches were now to be seen on the bright red arm. During the next twelve minutes the general hyperemia gradually subsided, and the special redness under and about the scratches again became visible. It was less pronounced than before and so too with the peripheral pallor; but the whealing continued undiminished. The last photograph was taken at this time (Fig. 5).

The temperature of the room was 21°C. throughout the period of the test.

Linear scabs formed later over the scratches, remaining in place for more than a week. There were never any signs of infection.

In this test Bier's spots developed first in the regions of the scratches, and where the latter were favorably placed the contraction of the small vessels was so complete as to empty them even to the margin of the lesions themselves (Fig. 6).

The several vascular reactions occurring about scratch injuries have been carefully considered by Lewis. Our scratches were deeper than those which he employed as routine, eliciting reactions more pronounced and lasting but otherwise differing no whit from those he

has described; for the differences referable to variations in the severity of the lesion are merely those of quantity not of quality, as he remarks. The "triple reaction" (wheal, flare, local vasodilatation) was characteristically present. In the analysis of our results we shall utilize both Lewis' terminology and the many new facts that he has placed at the disposal of investigators.

On consulting Figs. 3 and 4 it will be seen that Bier's spots appeared first, and were most numerous and pronounced, in the skin regions that exhibited vascular reactions as result of the scratching. The peripheral zone of pallor became much more extensive during the period when venous blood was accumulating in the limb (Figs. 1 and 2). This may have been because the partial vascular contraction responsible for it extended further than was perceptible prior to the venous engorgement. Whatever the actual cause the early appearance of Bier's spots in this zone was to have been expected, since partial emptying of the vessels is known to favor their occurrence (4). Blanchings also developed early nearer the lesions where the skin had shown the bright hyperemia, the "flare," due to a passive widening of the small vessels consequent upon arteriolar dilatation. This is not surprising since a preliminary active hyperemia is known to enhance the tendency to spotting (5). Later tests showed that in the regions of pallor and flare about histamine lesions spotting is likewise especially pronounced, doubtless for the same reasons. Both the vascular reactions mentioned are known to be the result of indirect stimulation through nerves, not of direct action of an "H substance" upon the vessels (6).

As Figs. 4 and 6 show, the blanching in some instances extended inwards secondarily, involving the entire whealed area immediately about the scratches. At first thought it would seem remarkable that any contraction could occur of vessels so damaged that the transudation of whealing had taken place from them. But Ebbecke (7) has shown that the period of transudation lasts for but a few minutes, the extravascular fluid remaining in place for hours afterwards. Doubtless this period was over by the time the Bier's spots developed.

There remains to be considered the "local reaction" of Lewis, the vasodilatation, that is to say, which is attributed to the direct effect of the "H substance." Under the conditions described in Test 1

this reaction could not be sharply discriminated from the flare. But in the case of even the slightest of scratches the local reaction develops; and, when the lesions have the severity of those now dealt with, it extends from 1 to 2 mm. about them, as our later work showed. Its precise dimensions were unimportant in the present instance since the blanching proved completely oblitative. The local pallor could be seen to extent gradually across the linear scratches where blood did not overlie them and prevent a clear view (Fig. 6).

The instance given is typical of the phenomena encountered in individuals prone to develop well marked Bier's spots. It leaves no doubt that the contractile impulse responsible for the spots is dominant over the influence which gives rise to local vasodilatation in skin mechanically injured.

The conditions in tissue chronically inflamed do not lend themselves readily to physiological analysis, being complicated by reparatory and other changes. The red or purplish hue of the skin is often due in no small part to new-formed blood vessels.

*Test 2.*—The subject S., a middle aged man, showed on the radial side of the right forearm just above the wrist, three curving, parallel injuries from 5 cm. to 12 cm. in length, where the skin had been raked by the claws of an animal about one week previously. The lesions now had the form of raised, edematous, purple strips some 3 mm. wide, each surmounted along its axis by a firm scab approximately  $\frac{1}{2}$  mm. thick and high. The contrast with the pale skin round about was great. No flare or pale areola could be seen about the injuries which appeared to be uninfected and healing. The edema and the purpling of the skin nearly coincided in extent.

The subject was placed at a table with the arm propped as usual and so turned that the scratches were uppermost. The pressure in the cuff above the elbow was raised to 70 mm. for 5 minutes and then to 170. Neither during the preliminary period of congestion nor that of stasis was a flare with peripheral pallor visible about the strips. During the 30 minutes of stasis Bier's spots appeared on the upper side of the arm but not on the lower. They were abundant and pronounced, but, as the microscope showed, nowhere so complete that all blood was forced out of them. Many intruded upon the purple strips which despite the general engorgement had remained well defined, being more deeply congested than the surrounding skin; and soon these no longer appeared as strips, so interrupted by blanchings were they. In not a few places the blanched spots, as they increased in size, involved the skin on the other side of the scab as if they had extended beneath it. Yet when one compared them as a whole with the blanchings in the uninjured tissue round about one found them not so white. Evidently some slight

impediment to complete vascular contraction existed within the region that had reacted to the injury.

With the removal of the cuff all the pale spots were lost in active hyperemia, the strips standing out intensely red. In another 20 minutes this had largely subsided, and the strips were turning violet. Throughout they had remained swollen.

The foregoing test illustrates the fact that the redness of chronic inflammation will yield on occasion to the blanching of Bier's spots. We have repeatedly observed them to encroach upon the scars of small incisions that had healed long previously by granulation. But such matters lie without the province of the present work. It suffices here to bring proof (as in Test 1) that the vascular contraction which leads to Bier's spotting prevails over the local vasodilatation which develops shortly after mechanical injury to the skin.

### *The Histamine Tests*

The histamine tests were carried out as follows:—

The preparation employed was the ergamine phosphate of Burroughs Wellcome and Co. When dissolved in saline and pricked into the arm according to Lewis' method it gave rise to the reactions described by this author. Sometimes it was introduced at situations where Bier's spots were known to be likely to occur in the individual utilized (Fig. 7). The highest effective dilution was in some individuals 1 in 500,000, and in others 1 in 200,000. When the arm was engorged with venous blood and the circulation cut off prior to the pricking a typical response was not always obtained even with the greater of the strengths mentioned, owing evidently, in some instances at least, to an oozing of blood from the congested skin, which must have swept out most of the histamine. To minimize this source of error several series of punctures were usually made at one time, together with control punctures of saline alone. They were placed in lines on the upper side of the horizontal forearm, which as usual was propped at elbow and wrist to facilitate the spotting. The maximum extent of the vascular reactions induced were sometimes marked out with dots of india ink.

In certain preliminary observations the histamine was punctured into the arm prior to cutting off the circulation, and the strength employed was not infrequently 1 in 1000, that is to say three times that used as routine by Lewis. It gave rise to pronounced "triple reactions." Ordinarily the Bier's spots appeared in the zones of flaring and pallor before they did in the unaffected skin, as was to

have been expected for reasons that have been sufficiently gone into; and sometimes the blanchings encroached slightly upon the wheals. Wheals, no matter how caused, tend to extend beyond the region of immediate transudation through the spread of the fluid composing them; while furthermore the vascular disturbance responsible for them is transitory, as has already been mentioned.

The essential point to be determined by the tests was whether the blanchings invaded the region of the local reaction of vasodilatation. As in the case of the scratches this merged so directly with the flare that its limits could not be defined. Therefore resort was eventually had to histamine punctures made after venous congestion and arterial occlusion had been brought about. Under such circumstances no wheal or flare develops, and the local reaction takes the form of a sharply defined purple disc with the puncture at its center. In numerous tests with graded dilutions of histamine, carried out upon several individuals, Bier's spots were never seen to intrude upon these characteristic discs though they often completely surrounded them, and proved capable of obliterating the purple dot due to control punctures of saline or to dilutions of histamine that failed to elicit the local reaction. In some of the cases studied the spotting occurred so soon after the punctures that it may well have limited the spread of the histamine within the skin (Fig. 7), the result being that the contractile impulse was opposed by that of said substance acting in a concentration which otherwise would not have obtained. However, against these instances can be placed others in which the blanchings threatened the histamine discs only after more than a half hour had elapsed, that is to say not until long after they had reached maximum dimensions. In tests upon one of the authors the circulation was sometimes cut off from the arm for 50 minutes.

It now became a crucial matter to determine the influence of Bier's spotting upon scratches laid down on the arm after venous congestion and arterial occlusion. For under such circumstances the local reaction due to the trauma might conceivably prove as obdurate as that resulting from histamine. Some evidence that this was not the case has just been mentioned, namely the obliteration by blanching of the purple dots due to puncture through saline or ineffective histamine dilutions. The following observations are conclusive.

*Test 3.*—This was made upon a middle aged man, R. In previous tests upon him histamine had proved ineffective in dilutions greater than 1 in 200,000, and this held true now when, as a preliminary, dilutions of 1 in 50,000, 1 in 100,000, 1 in 200,000 and 1 in 300,000 were pricked into the propped right forearm, about half way down on its flexor surface. Only the 1 in 50,000 gave a wheal larger than did saline alone, though the wheals resulting from 1 in 200,000 and 1 in 100,000 were somewhat more sharply defined than after saline. The 1 in 300,000 yielded a triple reaction no different from this last. After ten minutes the pressure was raised to 70 mm. Hg in a cuff on the upper arm, 5 minutes later to 170 mm., and immediately a second series of histamine punctures was laid down, this time on the uppermost side of the arm, in the skin overlying the radius. They were widely separated, and midway between each two a pair of scratches were made having the usual relations and dimensions. The row of lesions now extended from near elbow to near wrist, the interval between them averaging about 2½ cm. All this took 3½ minutes. A little more than a minute later the first slight mottling with Bier's spots had begun to appear here and there. Only the 1 in 50,000 and 1 in 100,000 histamine dilutions gave rise to the characteristic purple discs of vasodilatation and only that caused by the latter dilution became surrounded by Bier's spots during the 48 minutes of stasis. These spots had coalesced about it within 15 minutes, forming a uniform white background against which it stood out sharply; but the blanching never encroached upon it in the least. The local purpling around and between the parallel scratches, on the other hand, was replaced by blanching in several instances.

At once more extensive tests were undertaken on the left forearm, which was propped thumb up. The veins from the limb were first obstructed for 7 minutes at 70 mm. Hg pressure and then the arteries as well, at 170 mm. As rapidly as possible thereafter 5 series of histamine punctures were made on the radial side of the arm, with the 1 in 50,000, 1 in 100,000, 1 in 200,000 dilutions only, since these alone had previously proven effective. One series was laid down at a time in order of progressive histamine strengths and the needle was well cleansed before the next series was proceeded to. An assistant charted the situation of the punctures. The entire procedure took 3 minutes. Two series of the punctures were placed at about 4 cm. intervals in a line on the skin that was uppermost (that over the radius). The other series were located high on the flexor surface of the forearm, the punctures being somewhat closer together. Next, scratches were rapidly made in pairs between each two of the widely separated punctures over the radius and also beyond them at the wrist. This required another minute. There were seven pairs of scratches in all.

Bier's spots began to appear after the arterial occlusion had endured 7 minutes, but not in situations that threatened the histamine discs and the scratch reactions until after more than 15 minutes. By that time the spotting had crossed a tiny isthmus of undamaged skin existing midway in one of the scratches. Two small and faint discs had resulted from the 5 punctures with 1 in 200,000 histamine, 3

from the 5 with the 1 in 100,000 dilution, and 5 from 5 with the 1 in 50,000. These last were pronouncedly purple. No more developed during the succeeding 41 minutes before the circulation was restored to the arm after 56 minutes of stasis in all. During this time Bier's spots became numerous, large and brilliant. They merged and completely surrounded one of the two characteristic discs produced by the 1 in 200,000 histamine dilution but never encroached upon it; and they extended to the periphery of the other disc on one side without ever affecting its shape. The discs caused by the greater strengths of histamine likewise proved resistant to blanching. The local reaction next the scratches, on the other hand, which was manifest as an intense purpling between them and for approximately 2 mm. around them, had yielded to the blanching at the numerous places where this impinged upon it; and here and there the latter reached the scratches themselves and extended between them.

Special care was taken in this and other similar tests to determine whether the local reactions of histamine and mechanical injury tended to persist when the circulation once again entered the arm. On the present occasion both disappeared promptly in the reactive hyperemia.\* When the latter had largely subsided, after 21 minutes, a wide reddening was visible about the scratches and a whealing immediately next them. No histamine reactions whatever could now be discerned. Next day the scratches bore linear scabs and the tissue for a few mm. about them was hyperemic and edematous. Healing was prompt.

During the period of circulatory obstruction the engorged vessels in the areas of local reaction about the scratches had the same appearance under the binocular microscope as those in the histamine discs.

Important for the interpretation of such tests as this is the question whether the dilated vessels of the purple discs induced by histamine may not have proved obdurate to blanching because of an impacted content of red cells. For it is known that under ordinary conditions histamine causes small vessels to become clogged with cells, the plasma passing out into the tissues. But in instances such as the foregoing, in which the circulation was interrupted and whealing absent, there was no opportunity for corpuscles to accumulate in the dilated vessels by escape of plasma from them. They flushed promptly when blood was let into the arm.

\* It has been our experience that histamine diluted with Ringer's solution instead of saline is prone to produce discs which persist during reactive hyperemia and do not fade even when pressed upon with a glass slide when the hyperemia is at flood. Clotting must take place in the distended vessels. An intravascular clotting can also be produced by trauma, of course. Mere puncture with a needle sometimes gives rise to a purpling resistant to pressure and occasionally this was observed here and there along scratches such as we have employed.



The data obtained justify the conclusion that the local vasodilatation induced by minimal amounts of histamine resists the blanching of Bier's spots, whereas that caused by relatively severe mechanical injury in the same subject yields readily before it.

#### DISCUSSION

When pituitary extract or epinephrin is injected into regions of local vasodilatation due to acute inflammation no contraction of the vessels occurs (8); and yet, as we have shown, they yield before the contractile influence that is responsible for Bier's spotting. The fact is the more remarkable because this influence would seem to be but weak, proving ineffective ordinarily on the under side of the engorged arm, because the weight of the blood gravitating to the region acts to oppose it there (9).

The local vasodilatation produced by histamine is likewise refractory to pituitary extract or epinephrine (10) and this has been taken to corroborate the view that it is caused by the same "H substance" as the reaction at sites of inflammation (11). That the inference is unwarranted would seem to be sufficiently proven by the failure of the histamine reaction to yield before Bier's spotting. It may be objected that the tests with histamine involved the introduction of this substance from without, whereas the H substance of inflamed tissue is formed *in situ*. But the hypothesis of an H substance, in so far as it concerns histamine, rests on the basis of tests with this substance introduced from without. Furthermore in our experimental comparison of the resistance of the local reactions about scratches and histamine punctures, respectively, to Bier's spotting, all the conditions have been such as would in themselves tend to render the histamine reaction greatly the more susceptible. For the minimum effective quantity of histamine that was introduced by puncture, though persisting with undiminished effectiveness during short periods of stasis (12), must surely have been dissipated in part, by diffusion if in no other way, during the long periods of the present experiments. When the circulation was restored to the arm no whealing occurred at the site of puncture, nor any flare or local reaction after the reactive hyperemia had subsided. It is possible that this hyperemia may have flushed the histamine away or have lasted so long as to have masked

completely the transitory vasodilatation caused by the substance. With the scratch injury, however, no such difficulties of interpretation are met. Granting that the H substance causes the local reaction about the scratch, one must suppose either that this substance is formed in very large quantity to begin with or else continually in small amount; for otherwise the local reaction could not persist throughout many hours in an arm with good circulation, and survive prolonged hyperemia after stasis. On either supposition the local reaction due to the scratching should have proved the more obdurate to Bier's blanching. But always it yielded promptly whereas the histamine dilatation remained absolutely refractory.

Brief mention has been made of tests which showed that Bier's spots can invade old scars which have healed by granulation. Tests upon the vessels in new-formed granulation tissue may throw much light on the mechanism whereby the spots come about. The cutaneous pallor supervening upon death is due to a vascular contraction of the sort responsible for them. This pallor is said by morbid anatomists to involve granulation tissue as well as the normal skin; but the point deserves close study.

#### SUMMARY

Experiment shows that the vascular contraction responsible for Bier's spotting prevails over the local vasodilatation due to mechanical injury of the skin, and causes the local reddening to blanch. It is without effect, however, upon the local vasodilatation caused by histamine pricked into the skin. The results raise doubts concerning the validity of the hypothesis referring all local vasodilatations to the action of a single chemical substance or set of substances ("H substance"), liberated within the tissues.

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#### EXPLANATION OF PLATES

The pictures were all taken by Cooper Hewitt light. While giving strong contrast this emphasizes the red ingredient only of any violet hue that may be present.

#### PLATE 2

FIGS. 1-5. To show that the vasoconstriction of Bier's spotting prevails over the local vasodilatation caused by mechanical injury.

Parallel scratches just sufficiently deep to draw blood were made at five locations upon the right forearm. Fig. 1 shows the condition of affairs 20 minutes later. There is a spreading hyperemia with peripheral pallor and a well-marked wheal immediately about the scratches. Fig. 2 was obtained 5 minutes later, after the veins from the arm had been shut off for 3 minutes by 70 mm. pressure in a cuff on the upper arm. The hyperemia appears practically unchanged but the pallor round about it is more widespread. Pressure was maintained on the veins for another 4 minutes and then raised to 170 mm., cutting off the circulation entirely. Within the succeeding 12½ minutes Bier's spots became abundant in the regions of vascular reaction about the scratches, and extended even into the narrow space between scratches that lay parallel, as Fig 3 shows. For the purpose of this figure and later ones the camera was shifted so as to be almost directly above the partially supinated forearm. The photograph of Fig. 4 was taken after the circulation had been cut off for 23½ minutes. Pressure in the cuff on the upper arm was now relaxed. 12 minutes later, when the active hyperemia had largely subsided, Fig. 5 was obtained. It shows the whealing and local reaction that were noted prior to experiment, but the flaring hyperemia and pallor were less evident than before.

#### PLATE 3

FIG. 6. Enlargements of portions of Figs. 1, 4 and 5, to show the complete replacement by blanching of the vasodilatation next some of the scratches. Successive phases are here depicted of the reaction occurring about the two pairs of lesions that were highest on the arm, and hence most encroached upon by Bier's spots.

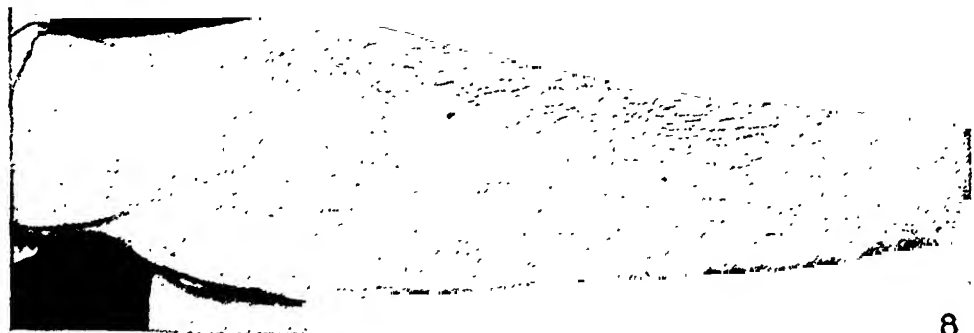
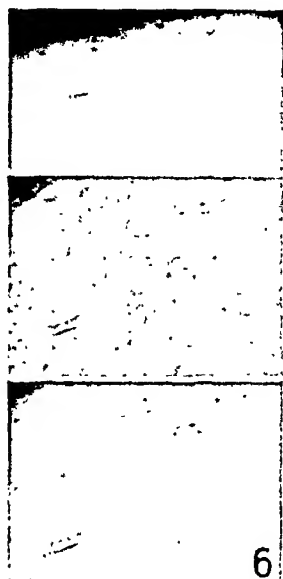
FIGS. 7 and 8. To show the obduracy of the vasodilatation induced by histamine. It had previously been found that the subject of this test was prone to develop a large Bier's spot over the radial basilic vein of the supinated forearm and a smaller one over the median basilic. Histamine in 1 in 1000 solution was pricked into the skin over the latter vein and seven minutes later a general venous obstruction was produced with a cuff on the upper arm. By this time an enormous wheal, with reddening and a flare, had developed about the needle puncture. 4½ minutes later the circulation to the arm was completely cut off and after another minute the histamine dilution was pricked into the skin over the radial basilic vein. Bier's spotting developed here almost as rapidly as did the purple histamine disc and soon it surrounded and sharply demarcated the latter. Fig. 7 was obtained after the circulation had been cut off 11 minutes, and Fig. 8 after 18 minutes more. The shape of the histamine disc had remained absolutely the same in the interval despite the progressive spread and intensification of the blanching about it. The test had only the use of furnishing a picture to illustrate the characteristic demarcation of histamine discs by Bier's spots. For not only was the amount of histamine pricked in several hundred times the minimum effective quantity but the rapid occurrence of vasoconstriction (Bier's spotting) about the disc while it was still developing must have served to retain the substance in concentrated form close to its original locus.





(Rous and Gilding: Cause of local vasodilatation)





(Rous and Gilding: Cause of local vasodilatation)





# AN APPARATUS FOR THE STUDY OF RESPIRATORY QUOTIENT AND BASAL METABOLISM OF MICE

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During the course of some investigations in this laboratory, it became necessary to ascertain the respiratory quotient and basal metabolism of mice. Therefore, a study was made of an apparatus and procedure which could be readily applied to a large number of animals, and would yield precise results.

Apparatus for determining respiratory quotients fall into two classes, the open-circuit type and the closed-circuit type. In the former, a stream of dry air freed from carbon dioxide is passed through a chamber containing the animal. The water vapor and carbon dioxide in the outgoing air are absorbed and weighed. At the same time, the loss in weight of the animal and the weight of its container are noted. The amount of oxygen used is then determined indirectly as the difference between the combined weights of water and carbon dioxide given off and the loss in the weight of the animal. Considerable precaution must be taken in weighing the absorption vessels and animal chamber. Haldane<sup>1</sup> was among the first to apply this procedure to the study of respiratory exchange of small animals, so that the method is commonly known by his name. This apparatus was also used by Pembry<sup>2</sup> in studying the rate of respiration of mice at various temperatures, and by Moog<sup>3</sup> for determining the respiratory quotient of guinea pigs. It has been somewhat modified and greatly complicated by Murschauser,<sup>4</sup> who removed the carbon dioxide by means of an elaborate system of absorption bottles containing barium hydroxide solution, and determined the oxygen consumed by analysis of samples of the expired air. More recently, Aszodi<sup>5</sup> has used a simple apparatus of this type for the study of induced hibernation in white mice. Several experiments in which the oxygen consumed was ascertained directly as well as indirectly showed that the values obtained by the two methods agreed to within 2

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<sup>1</sup> Haldane, J. S., *J. Physiol.*, 1891, 13, 419.

<sup>2</sup> Pembry, M. S., *J. Physiol.*, 1893, 15, 401.

<sup>3</sup> Moog, R., *Compt. rend. Soc. biol.*, 1911, 71, 520.

<sup>4</sup> Murschauser, H., *Biochem. Z.*, 1912, 42, 262.

<sup>5</sup> Aszodi, Z., *Biochem. Z.*, 1921, 113, 70.

per cent. Aszodi's apparatus has been applied to the metabolism of white rats by Goto,<sup>6</sup> Asada,<sup>7</sup> and Händel and Tadenuma.<sup>8</sup> A very simple continuous-flow apparatus has been described by Artundo,<sup>9</sup> for use with rats and rabbits in which the respiratory exchange is determined by analysis of samples of the expired air by means of a Haldane gas analysis apparatus.

In the closed-circuit type of apparatus, the respired air is passed through an absorbent to remove the carbon dioxide and then returned to the respiration chamber to be used over again. Oxygen is supplied as needed to replace that taken up by the animal. Thus, the oxygen consumed is measured directly by the quantity required to maintain constant pressure in the system. The amount of carbon dioxide given off is obtained by weighing the absorption vessels or by titration. An apparatus of this type was first applied to the study of small animals by Regnault and Reiset.<sup>10</sup> Zuntz<sup>11</sup> has described an exceedingly complicated apparatus operating on this principle which was later modified by Zuntz and Oppenheimer.<sup>12</sup> Elsas<sup>13</sup> studied the respiratory exchange of frogs, using an apparatus in which air was circulated by a motor-driven pump, and the carbon dioxide absorbed in barium hydroxide solution was determined by titration. The oxygen was measured as introduced from a gasometer and checked by analysis of samples of the respired air. The experimental period extended over 20 to 24 hours. He considered the average error to be about 2 per cent. The same apparatus was used by Lesser<sup>14</sup> for the determination of the respiratory quotients of mice. He reported that the error in the quotients obtained did not exceed 0.02. Hildebrandt<sup>15</sup> used a similar apparatus for the study of the respiration of white rats. A model described by Benedict<sup>16</sup> for use with rabbits and guinea pigs includes special provision for recording movement of the animal and for introducing food. It is designed for use over a period of 24 hours or longer. A very elaborate apparatus for experiments lasting a week or more has been constructed by Kolls and Lovenhart.<sup>17</sup> Rubner<sup>18</sup> has described a comparatively simple device for use with animals weighing from 20 to 60 grams. Air is circulated by means of a rotary

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<sup>6</sup> Goto, K., *Biochem. Z.*, 1923, 135, 107.

<sup>7</sup> Asada, K., *Biochem. Z.*, 1923, 143, 387.

<sup>8</sup> Händel, M., and Tadenuma, K., *Z. f. Krebsforschung*, 1924, 21, 197.

<sup>9</sup> Artundo, A., *Compt. rend. Soc. biol.*, 1927, 97, 407.

<sup>10</sup> Regnault, V., and Reiset, J., *Ann. Chim. Phys.*, 1849, 26, 299.

<sup>11</sup> Zuntz, N., *Arch. f. Physiol., Suppl.*, 1905, p. 431.

<sup>12</sup> Zuntz, N., and Oppenheimer, C., *Biochem. Z.*, 1908, 14, 361.

<sup>13</sup> Elsas, B., *Z. f. Biol.*, 1913, 62, 3.

<sup>14</sup> Lesser, E. J., *Biochem. Z.*, 1924, 153, 39.

<sup>15</sup> Hildebrandt, F., *Arch. exp. Path. u. Pharm.*, 1922, 92, 68.

<sup>16</sup> Benedict, F. G., *J. Biol. Chem.*, 1915, 20, 301.

<sup>17</sup> Kolls, A. C., and Lovenhart, A. S., *Am. J. Physiol.*, 1915, 39, 67.

<sup>18</sup> Rubner, M., *Sitzb. preuss. Akad. Wiss.*, 1924, p. 2.

pump. Oxygen is supplied by electrolysis, the volume added being determined by measuring the volume of hydrogen evolved at the cathode. The duration of an experiment is about 24 hours. Other apparatus for use with small animals have been described by Foster and Sundstroem,<sup>19</sup> Wesson,<sup>20</sup> and Schoeller, Gehrke, and Michael.<sup>21</sup> Fridericia<sup>22</sup> has described a closed-circuit apparatus in which the oxygen consumed can be determined by two methods, thus obtaining a double check on this value. Cori and Cori<sup>23</sup> have used the apparatus of Fridericia in a study of the metabolism of rats. Agreement between the values of direct and indirect oxygen determinations averaged 1.4 per cent. They consider the value for the respiratory quotient to be accurate to  $\pm .008$ .

Although many of the apparatus cited above are applicable to the study of the metabolism of mice, none are particularly adapted to rapid routine manipulation. The difficulties encountered in keeping a large circulatory system perfectly air tight have been emphasized by many workers. Any procedure involving methods of exact gas analysis requires particular care and is very time consuming, as is true of methods involving accurate weighing of gas absorption vessels. An attempt to develop a procedure which will avoid these disadvantages, and at the same time provide a method for the measurement of the respiratory quotient with sufficient accuracy, has resulted in the apparatus and technique described in this paper.

The apparatus (Fig. 1) consists of a closed glass chamber (A) of about 500 cc. capacity, in which a wire cage (B) is suspended. The carbon dioxide given off by the animal is absorbed by an N/20 solution of barium hydroxide. This solution is introduced into the chamber from burette (C) at the beginning of the experiment. At the conclusion of the experiment, the excess barium hydroxide is titrated with an N/50 solution of oxalic acid contained in burette (D). An air-propelled fan and glass paddle mounted on the same shaft circulate the air and thoroughly stir the liquid. The shaft of the stirrer enters the chamber through a mercury seal (E). The chamber is ventilated before and after the experiment by aspirating a current of air through tubes (F) and (G). Variations in the pressure during the run are

<sup>19</sup> Foster, G. L., and Sundstroem, E. S., *J. Biol. Chem.*, 1926, 69, 565.

<sup>20</sup> Wesson, L. G., *J. Biol. Chem.*, 1927, 73, 499.

<sup>21</sup> Schoeller, W., Gehrke, N., and Michael, S., *Biochem. Z.*, 1927, 189, 220.

<sup>22</sup> Fridericia, L. S., *Biochem. Z.*, 1913, 54, 92.

<sup>23</sup> Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1926, 70, 557.

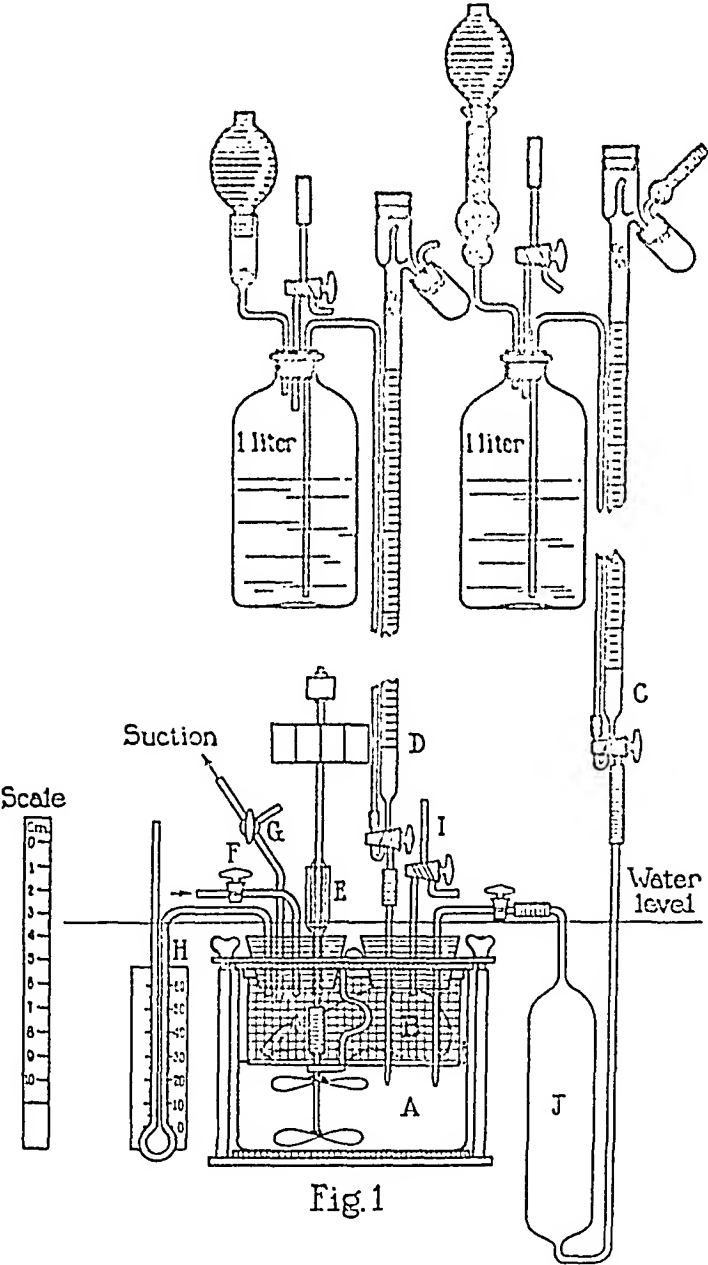


Fig. 1

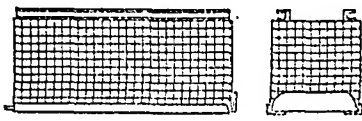


Fig. 2

recorded on the manometer (II). When the chamber is closed, the animal consumes oxygen and the pressure in the chamber is reduced. Compensation is made by running in an equivalent volume of barium hydroxide solution so that the amount of oxygen consumed is measured by the volume of barium hydroxide solution required to maintain atmospheric pressure within the chamber. The volume of carbon dioxide given off during the same period is calculated from the titration. The respiratory quotient is then obtained by dividing this volume of carbon dioxide by the volume of oxygen consumed. Proper functioning of the apparatus can be checked at any time by running a blank experiment in which a measured amount of pure, dry carbon dioxide is passed into the chamber. Capillary stop-cock (I) is provided for this purpose. The brass plate which carries the cage, together with all inlets and outlets to the chamber, is fixed rigidly to an iron stand which also supports the burettes and reservoirs. The removable wire cage (Fig. 2) is provided with a sliding floor for the introduction of the animal. The glass chamber rests on a brass plate which is clamped to the fixed plate by thumb screws. The fixed plate carries a rubber gasket which insures an air-tight seal. The chamber and reservoir (J) are immersed in a water thermostat. The purpose of this reservoir is to eliminate temperature changes in the barium hydroxide solution when it is introduced into the chamber. Absolute quiescence of the animal throughout the experiment is assured by an intraperitoneal injection of "luminal sodium."

### *Experimental Procedure*

The animal is injected intraperitoneally with 0.10 cc. of a 2 per cent solution of "luminal sodium," and immediately placed in the wire cage which is then suspended under the fixed plate. Since, during the experiment, the air of the chamber is in contact with a dilute aqueous solution, provision must be made for saturating it with water vapor during the preliminary period. Otherwise the vapor pressure, which would develop as soon as the chamber should be closed, would introduce error into the oxygen determination. To this end, 5 cc. of water are placed in the chamber together with a few drops of phenolphthalein indicator necessary for the subsequent titration. The chamber is then clamped firmly into place. The fan is started, and the suction adjusted so as to provide a current of air at the rate of about 1 liter per minute. For the reasons just mentioned, the air used for ventilation must not only be at the same temperature as the thermostat but must also be saturated with water vapor. The incoming air, therefore, circulates

through a copper coil and humidifying device immersed in the bath. The temperature and humidity of the incoming air may easily be checked by closing the chamber and observing any changes in the manometer. About 30 minutes are required to reach thermal equilibrium. However, the actual run is not started until one hour has elapsed from the time of the injection, so that the animal will be thoroughly under the influence of the drug. With the current of air still passing through the chamber, about 30 cc. of the barium hydroxide solution are introduced, stop-cock (G) is turned to divert the suction to the open air, and stop-cock (F) is closed, thus sealing the chamber. The time of closure is recorded and the barium hydroxide burette is read. As the oxygen is consumed by the animal, the pressure in the chamber decreases, as indicated by the manometer. Barium hydroxide solution is run in as required to restore atmospheric pressure. At the end of about 30 minutes, the time is again recorded, stop-cock (F) is opened, suction is restored, and the excess barium hydroxide titrated rapidly with oxalic acid. The volume of oxygen consumed is given directly by the volume of barium hydroxide solution introduced during the run. From the titration data, the temperature of the bath, and the barometric pressure, the volume of carbon dioxide given off by the animal is computed. Data and calculations from a typical experiment follow.

Weight of animal.....	23.3 gm.	Normality of Ba(OH) <sub>2</sub> ....	0.0491
Temperature of bath .....	28°C.	Normality of H <sub>2</sub> C <sub>2</sub> O <sub>4</sub> .....	0.0204
Barometric pressure.....	760 mm.	Duration of experiment...	28 minutes

Ba(OH) <sub>2</sub> introduced at start.....	30.00 cc.
Ba(OH) <sub>2</sub> added to compensate for oxygen consumed.....	21.55 cc.
Total Ba(OH) <sub>2</sub> added.....	51.55 cc.

Volume of H <sub>2</sub> C <sub>2</sub> O <sub>4</sub> equivalent to 51.55 cc. of Ba(OH) <sub>2</sub> .....	123.72 cc.
“ “ H <sub>2</sub> C <sub>2</sub> O <sub>4</sub> required to titrate excess Ba(OH) <sub>2</sub> .....	59.35 cc.
“ “ H <sub>2</sub> C <sub>2</sub> O <sub>4</sub> equivalent to CO <sub>2</sub> absorbed.....	64.37 cc.

Volume of CO <sub>2</sub> absorbed at standard conditions as calculated from above.....	14.64 cc.
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Volume of CO <sub>2</sub> absorbed at 760 mm. and 28°C.....	16.14 cc.
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Volume of O <sub>2</sub> consumed at 760 mm. and 28°C. (vol. Ba(OH) <sub>2</sub> added) ..	21.55 cc.
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$$\text{Respiratory quotient} = \frac{16.14}{21.55} = 0.749$$

### *Standardization of the Apparatus*

The accuracy of the apparatus is dependent upon the extent to which the carbon dioxide absorbed represents the carbon dioxide given off by the animal during the time of the run. The degree to which this condition is fulfilled may be tested by introducing a known

volume of pure carbon dioxide under circumstances which simulate as nearly as possible the conditions of the experiment.

TABLE I  
*Standardization of Apparatus by Means of Pure Carbon Dioxide*

Experiment No.	Rate of introduction of CO <sub>2</sub> <i>cc. per minute</i>	Volume of CO <sub>2</sub>	
		Introduced	Titrated
		<i>cc.</i>	<i>cc.</i>
1	0.47	15 00	14.91
2	0.48	15 00	15.06
3	0.51	15 30	15.24
4	0.8	20 00	20.20
5	0.8	20 00	20.20
6	1.1	16 20	16.70
7	1.8	21 00	21.50
8	1 9	20 00	20.20
9	3 2	20 00	19.25

TABLE II  
*Respiratory Quotients of Fasted Mice*

Exp. No.	Animal	Weight	Duration of run	O <sub>2</sub> consumed	CO <sub>2</sub> evolved	R. Q. CO <sub>2</sub> /O <sub>2</sub>
		<i>grams</i>	<i>minutes</i>	<i>cc.</i>	<i>cc.</i>	
1	1	19.30	37	20.00	14.10	0.705
2	1	19.30	33	20.60	14.35	0.716
3	2	25.50	33	20.20	13.90	0.688
4	2	25.50	37	20.20	14.27	0.706
5	3	28.60	31	20.30	14.10	0.694
6	3	28.60	33	20.55	14.85	0.723
7	4	26.40	27	20.60	14.32	0.696
8	4	26.40	31	20.05	14.50	0.725
9	5	20.50	34	20.00	14.17	0.708
10	5	20.50	35	20.25	14.04	0.695
11	6	22.00	36	20.05	14.62	0.729
12	6	22.00	35	20.00	14.62	0.731
Average .....						0.710

The apparatus is arranged in the thermostat and a stream of carbon dioxide from a gas burette is passed into the chamber through the capillary stock-cock (I). The rate of introduction of the gas is adjusted to duplicate as nearly as possible



the rate at which carbon dioxide is normally expired by a mouse, as determined in previous experiments. In order to be certain that variations in the rate of production of carbon dioxide, which might be expected in normal animals, were without appreciable effect upon the validity of the data, carbon dioxide was introduced at rates varying from 0.5 to 3 cc. per minute. The stream of carbon dioxide is allowed to pass into the chamber during a preliminary period of about 20 minutes. 50 cc. of barium hydroxide solution are run into the chamber, this quantity being approximately the total amount used in a determination upon an animal. The chamber is then closed. The stream of carbon dioxide is continued until about 15 to 20 cc. have been introduced. Ventilation is reestablished and the excess barium hydroxide titrated. A comparison of the carbon dioxide absorbed with the carbon dioxide introduced, as read on the gas burette, serves as an indication of the accuracy of the apparatus. Data obtained in this manner are given in Table I.

The operation of the apparatus was further checked by determining the respiratory quotients of mice which had been fasting for 36 hours. It has been shown that fasted rats give a respiratory quotient of 0.71 to 0.72, and Benedict<sup>24</sup> has recommended their use for the standardization of apparatus employed in the study of metabolism of small animals. Duplicate runs were made in each case. Data obtained are given in Table II.

#### DISCUSSION

The apparatus and procedure here outlined are, in some respects, distinct departures from the methods heretofore employed. In the first place, the animal is confined in a chamber where the atmosphere is saturated with water vapor. Although it has been shown<sup>25</sup> that above 25°C. the effect of increased humidity is to raise the metabolic rate, there is little reason for believing that it would have any pronounced effect on the respiratory quotient. Values for the respiratory quotient of normal animals determined in this apparatus appear to confirm this inference.

In other apparatus of this type, the composition of the air is kept practically constant throughout the experiment by introducing pure oxygen to replace that consumed. This necessarily involves complications in the apparatus which can be avoided if the oxygen content be allowed to decrease within certain limits. It has already been

<sup>24</sup> Benedict, F. G., in Abderhalden, E., *Handbuch der biologischen Arbeitsmethoden*, Berlin, 1926, Abt. IV, Teil 10, 537.

<sup>25</sup> Cluzet, J., *Compt. rend. Acad.*, 1908, 146, 773.

shown<sup>26, 27, 28, 29</sup> that a decrease in the oxygen content of respired air is without noticeable effect upon respiration as long as it does not fall below 10 to 12 per cent. In the present procedure, the concentration of oxygen in the chamber is never less than 15 per cent, so that the metabolism of the animal should at no time be affected by oxygen want.

The success of the carbon dioxide determination depends upon the extent to which the amount of carbon dioxide, computed from the titration, represents the amount of carbon dioxide given off by the animal during the time of the experiment. If air is passed through the chamber at the rate of one liter per minute, the concentration of carbon dioxide, initially present, will be from 0.5 to 1 cc. per liter. Experiments in which pure carbon dioxide was introduced into the chamber indicated that with this initial concentration the carbon dioxide was taken up by the barium hydroxide as fast as it was introduced, so that, at the close of the run, the concentration of carbon dioxide was practically unchanged. Therefore, the carbon dioxide present at the beginning is compensated for by the same amount of carbon dioxide left unabsorbed at the end of the run. This being the case, the carbon dioxide absorbed will be an accurate measure of the carbon dioxide given off by the animal during the time of an experiment.

The volume of the chamber necessarily imposes certain limits upon the total amount of fluid introduced. Although the accuracy of a titration is favored by the use of dilute solutions, it is essential that the barium hydroxide solution be of a sufficient concentration to produce rapid absorption of carbon dioxide. It was found that an N/20 solution best fulfilled these requirements. The limited space available for titration did not make it feasible to use an acid solution of a greater dilution than N/50.

The accuracy of the carbon dioxide determination will be affected by the speed at which the barium hydroxide solution is introduced at the beginning of the run, and the time required to titrate the excess

<sup>26</sup> Friedländer, C., and Herter, E., *Z. A. physiol. Chem.*, 1879, 3, 19.

<sup>27</sup> Fraenkel, A., and Geppert, J., *Compt. rend. Acad.*, 1883, 96, 1740.

<sup>28</sup> Terray, P., *Pflüger's Arch.*, 1896, 65, 393.

<sup>29</sup> Durig, A., *Arch. f. Physiol., Suppl.*, 1903, p. 209.

barium hydroxide at the end of the run. However, by the exercise of reasonable precaution, error from this source should be negligible.

From a study of the data in Table II, it appears that the probable error of a single observation is 0.015, and the probable error of the arithmetical mean is 0.004. These data, together with the results obtained in the standardization of this apparatus with pure carbon dioxide, indicate that the values for the respiratory quotients obtained are accurate within 0.02. The mean value obtained from a series of such observations should be readily brought to an accuracy of 0.01.

#### CONCLUSIONS

A simple apparatus has been developed for the study of the respiratory quotient and basal metabolism of mice. Data are given which indicate that the values for the respiratory quotient obtained are accurate to within 0.02. The apparatus is especially designed for rapid, routine manipulation.

# THE EFFECT OF UNILATERAL NEPHRECTOMY ON THE SENILE ATROPHY OF THE KIDNEY IN THE WHITE RAT

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Arataki (1) has demonstrated that there is a decrease in the number of glomeruli in the kidney of the white rat in old age. According to Arataki (1) this senile loss of glomeruli begins after 350 days of age and reaches two-thirds of the adult value at 500 days of age. Since this atrophy is not accompanied by gross or histologic evidence of pathologic destruction of the glomeruli, we may tentatively consider it as physiologic.

Arataki (2), Jackson and Shiels (3) Moore (4) and others have demonstrated that unilateral nephrectomy during the growth period (1 to 150 days of age) has no effect on the total number of glomeruli in the adult kidney. We have found no published reports concerning the effect of unilateral nephrectomy on senile atrophy.

## *Method*

Thirty-two white rats from the colony of the Dept. of Zoology of Yale University were used, and are designated as the Yale Strain.† All animals were 200 days of age at the beginning of the experiment. The right kidney was removed from 16 animals by the usual lumbar route, under ether anesthesia. The remaining 16 animals were used as controls. Two animals from each group were sacrificed by a blow on the head at intervals of 30 days and the left kidney was injected with Janus Green B according to the technique of Nelson (5). The right kidney of the control animals was fixed in Zenker formol, embedded in paraffin and sectioned for histologic examination and measurement of glomeruli.

The total number of glomeruli in the injected kidneys was counted according to the technique of Nelson (5). In all cases the counts are complete enumerations of the left kidney.

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† This designation is necessary, since in the quantitative studies on the kidney carried on in this laboratory, three strains of white rats have been used.

TABLE I  
*Nephrectomy at 200 Days of Age*

Experimental			Control		
Rat No.	Count	Weight	Rat No.	Count	Weight
Sacrificed 230 days (Dec. 1-1928)					
		gms.			gms.
3	28,756	195	23	28,092	170
4	41,427*	215	18	40,369*	216
Sacrificed 260 days (Jan. 1-1929)					
1	28,142	195	20	24,299	193
2	26,000	182	17	25,745	185
Sacrificed 290 days (Feb. 1-1929)					
5	20,569	245	22	21,693	253
6	25,121	223	25	26,491	223
Sacrificed 320 days (Mar. 1-1929)					
7	18,549	248	24	17,272	225
8	22,687	200	29	21,541	200
Sacrificed 350 days (April 1-1929)					
9	21,870	210	27	24,093	210
10	22,333	205	18	24,538	205
Sacrificed 380 days (May 1-1929)					
11	22,723	185	26	22,591	185
12	21,504	185	19	22,526	200
Sacrificed 410 days (June 1-1929)					
13	17,727	153	30	23,727	157
14	28,967*	195	28	24,227	200
Sacrificed 440 days (July 1-1929)					
15	29,889*	158	32	22,939	158
16	21,725	180	31	22,116	177

\* These animals are of a different strain. See discussion in the text.

## RESULTS

The results may be divided into two series of observations, first, the total glomerular counts, and second, glomerular sizes.

Inspection of Table I and Fig. I reveals that control animals with a count of about 28,000 at 230 days of age gradually decline to a count of 18,000 to 22,000 at 320 days of age. After 320 days of age there is little further loss of glomeruli. This is in general agreement with the results of Arataki (1).

It is further quite evident that there is no significant variation between the control and experimental animals. The experimental method precludes any quantitative observations on increase in

TABLE II

Age	Size of glomeruli (microns)		
	Minimum	Maximum	Average
<i>Days</i>			
230	70	123	95.2
440	38	157	103.6

TABLE III

Age	Average diameter of glomeruli (microns)	Total number of glomeruli	Total glomerular surface mm. <sup>2</sup> ( $4\pi r^2$ = surface)
Adult.....	95.2	28,092	799.7
Senile.....	103.6	22,527	759.6

weight, but on inspection, the left kidney of the experimental group was appreciably larger than the controls. These results force the conclusion, that despite increase in size, unilateral nephrectomy during adult life has no effect on the senile atrophy of the opposite kidney as expressed in renal units.

Variations in the size of glomeruli were determined by measuring in Zenker fixed, paraffin sections, the diameters of 200 glomeruli at hilus level, in one adult and one senile kidney. The results are shown in Table II and plotted as frequency curves in Fig. II. The glomeruli in the senile kidney average 8.5 microns larger and show greater

variation in size. If the total surface of the visceral layer of Bowman's capsule is calculated, (Table III) it is found that the loss in renal units is accompanied by an increase in size. That this glomerular enlargement is a true hypertrophy, is not apparent at this time, since there is no evidence that the larger glomerulus has a greater functional capacity. The finding of a greater variation in the size of the glomeruli in senile atrophy is not in harmony with the results of Karsner, Saphir and Todd (7) in cardiac atrophy. In an atrophic heart,

NUMBER OF GLOMERULI FIG. I - TOTAL GLOMERULI AT DIFFERENT AGES

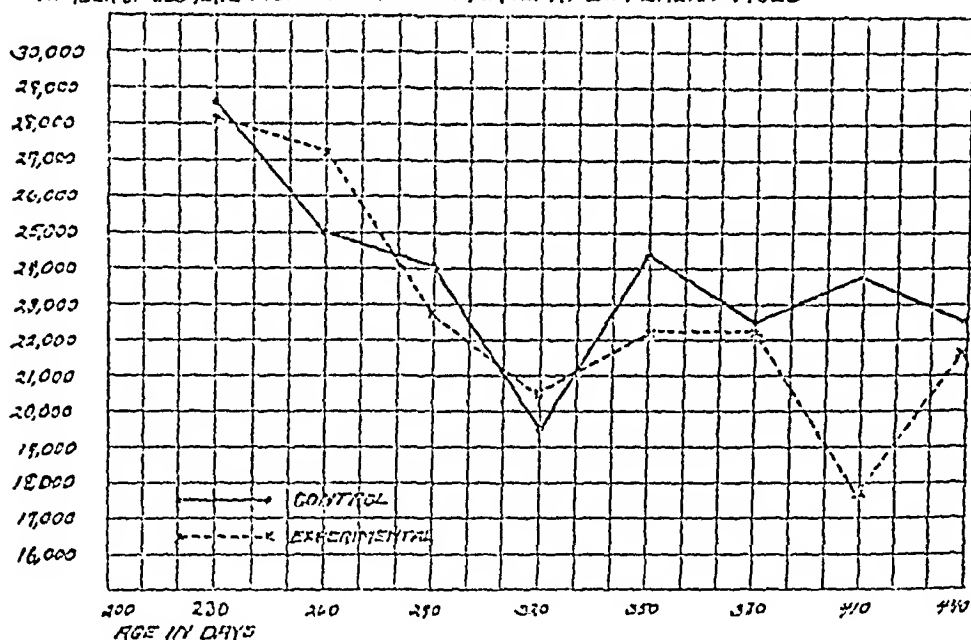


FIG. I

associated with tuberculosis, they found a greater uniformity in the size of muscle fibers than in a normal heart. This variation of morphology in two types of atrophy does not support the hypothesis of Bradley (8) of a single pathogenesis of decreased blood supply.

The method of disappearance of glomeruli in old age is interesting. As yet we have been unable to follow the process. Our present observations are confined to a complete serial section of the right kidney of a rat 440 days of age. In these sections there are insufficient fibrotic glomeruli to account for the total loss and we are inclined to

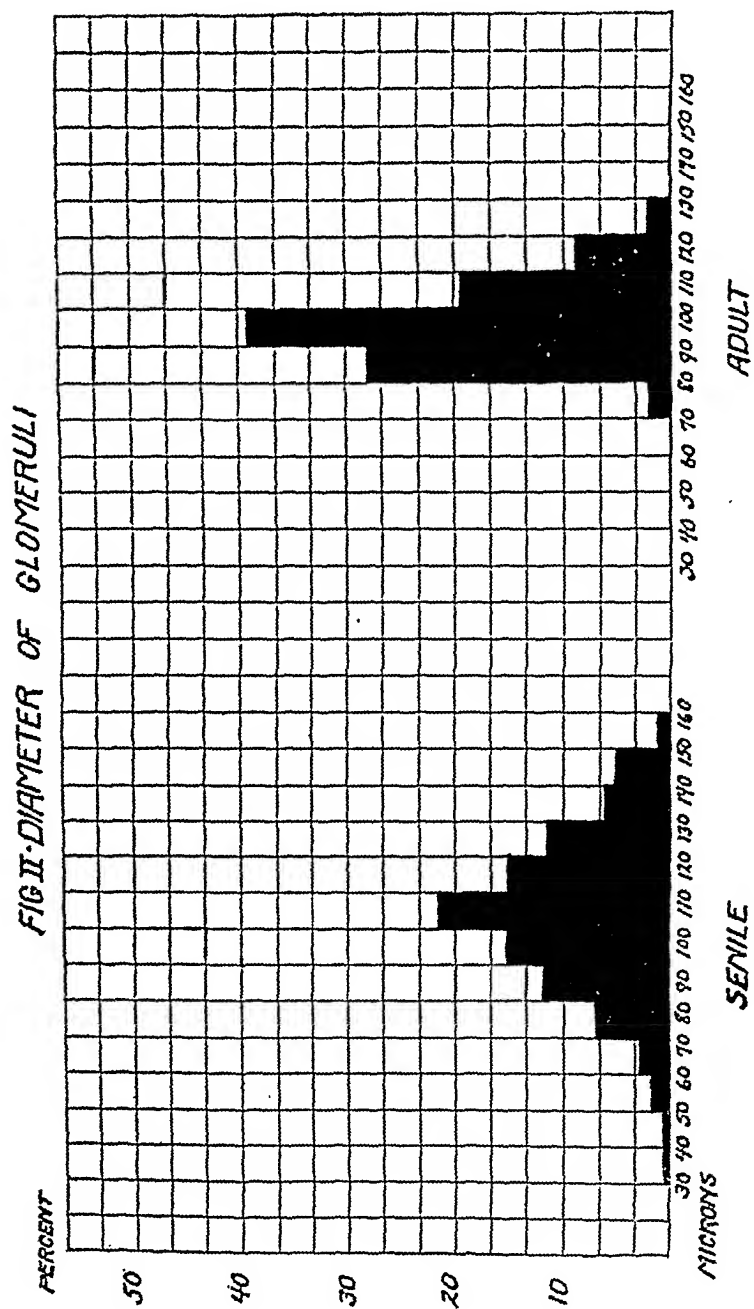


FIG. II



accept a process of gradual atrophy and complete disappearance as proposed by Chase (6) in *Necturus*.

The counts on rats No. 4, 14, 15 and 18 are worthy of further comment. The rats designated here as the Yale strain are a mixture of the Wistar rat and an ordinary wild rat. It is evident that this Yale strain is a hybrid stock composed of two substrains, one with 40,000 and the other with 30,000 renal units. This finding together with the possible effect of heredity on the total number of glomeruli is under investigation at the present time.

#### DISCUSSION

The results here reported have significance in the interpretation of the remote effects of unilateral nephrectomy in man. The investigation of Hayman and Starr (9) and Moore and Lukianoff (10) have demonstrated that the fluid output of the kidney is dependent in general on the total number of open glomeruli and this latter factor is limited by the total glomerular units in the kidney. If we postulate that approximately 25% of the glomerular filtration space is necessary for basal demands, it follows that the removal of one kidney during adult life leaves a reserve of 100%, but as this same animal approaches senility, further glomerular units are lost and a reserve of only 33% remains. Since the kidney of man undergoes analogous senile changes, it may be safely assumed that a patient under similar circumstances, would develop renal insufficiency after a kidney injury, innocuous to a patient with both kidneys.

The conclusions arrived at in this paper add quantitative support to the doctrine that "old age is inevitable." The senile loss of glomeruli proceeds at the usual rate and to the same extent despite the absence of one kidney. The relations between the total glomerular units and the number of open glomeruli in senile kidneys and the changes in circulation dependent on senility are under investigation in this laboratory.

Arataki (1) found that senility in the Wistar rat occurred after 350 days of age, while our observations place the time at about 300 days. It is possible that senility occurs at different ages in different strains of the same species. Unpublished observations from this laboratory indicate a wide variation in the age period of senile glomerular loss in the human kidney.

## CONCLUSIONS

1. In senility in the white rat there is a decrease in the total number of glomerular units.

2. The decrease in glomerular units is associated with an increase in the average diameter and a greater variation in size, of the remaining glomeruli.

3. Unilateral nephrectomy during adult life has no effect on these senile changes.

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# ON THE ISOLATION, CULTIVATION AND CLASSIFICATION OF THE SO-CALLED INTRACELLULAR "SYMBIONT" OR "RICKETTSIA" OF PERIPLANETA AMERICANA

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PLATE 4

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## 1. INTRODUCTION

The bacteria-like so-called intracellular "symbionts" or "rickettsiae" found in some arthropods have been proven to be transmitted and to be associated with the production of fatal diseases in man and in domestic animals. To the presumably pathogenic forms belong those associated with exanthemic fever, trench fever, Rocky Mountain spotted fever, and heartwater. Dengue fever and some other arthropod-transmitted diseases, such as the Japanese flood fever, are also suspected to be associated with "rickettsiae." One "rickettsia" (*R. melophagi*) is transmitted by its vector (*Melophagus ovinus*) to sheep in which it apparently survives as a harmless parasite. The majority, however, are at present not transmitted to higher animals, but usually exist within certain specialized cells of invertebrates and are passed from one generation to another through their eggs.

Very little is known about either the pathogenic or the non-pathogenic intracellular parasites under discussion because it has so far been impossible to adapt most of them to artificial media. Some claim that the parasites are bacteria (1); others that they should be placed among the recent so-called "rickettsiae" (2) a group erected by da Rocha-Lima in 1916, for certain small microorganisms found in the human body louse infected with the virus of typhus fever. A third class of investigators has concluded that the intracellular inclusions are not microorganisms at all, but products of cellular metabolism (3). Some of the structures observed might represent bacteria; others a totally new type of microorganism; and lastly, still others might, conceivably, represent products of cellular metabolism.

Many arthropods apparently harbor more than one species of "symbiont." In some cases a form pathogenic to vertebrates exists together with a non-pathogenic form. Frequently the two forms resemble one another so closely, morphologically, that cultures become imperative in order to differentiate them, as Noguchi showed in 1926 (4). The cultural possibility also opens up a number

of interesting biological problems among which the much discussed question of symbiosis has prominence.

In 1920 the writer (5) assumed that he had cultivated a spirillum from the bacteriocytes of *Parcoblatta virginica*, and one from *Periplaneta americana* caught near Boston. These spirilla in some of their morphological characters resembled the intracellular parasites of the roaches. However, these results were apparently due to certain technical pitfalls not very evident at that time (6).

## 2. Location and Morphology of the Intracellular Parasite

In every individual of *Periplaneta americana*, the large American roach, the bacteriocytes (mycetocytes) are disseminated throughout the fat tissue. Fresh preparations or stained films and sections of fat tissue (at 100 diameters magnification) show the bacteriocytes to be independent, single, irregular cells embedded among fat cell groups. These bacteriocytes possess distinct nuclei and their cytoplasm is packed with granular material. This material resolves itself into definite shapes at magnifications of from 900 to 1800 diameters. Bacteriocytes, excised and triturated in normal salt solution, liberate the cytoplasmic inclusions which appear bacteria-like in form. The elements are not motile and are often congregated into groups. In sections the intracellular units are stained with difficulty. We have obtained satisfactory results by subjecting sections of roaches cut at 8 and 10 $\mu$  to a prolonged treatment with Delafield's hematoxylin.

The parasites, liberated by triturating, are very easily stained and are found to be non-encapsulated, Gram-positive and not acid-fast. Smears fixed while wet in alcohol-ether for 15 minutes and stained for 15 to 20 minutes according to Giemsa, give pictures as illustrated in Fig. 1. The microorganisms are pleomorphic, showing equal and unequal division or budding. Two equal or unequal adhering units are common, and three such units are also seen. Coccoidal, short and longer straight or curved bacillary forms are in evidence. These bacilli often terminate in a thickening. The microorganisms may stain uniformly with Giemsa, but frequently assume a banded, granular or bipolar arrangement of the chromatic material. Spores have never been observed. The individual units of the bacillary forms found within the bacteriocytes range in length from 2 to 5 $\mu$  with a mean length of 3 $\mu$ . Their breadth varies from 0.5 to 1 $\mu$ . Three adhering units may total 8 $\mu$  in length and coccoidal or bud forms

measure from 0.5 to 1  $\mu$  in diameter. These facts taken into consideration with those previously noted, that the species does not form capsules, is not motile, not acid-fast, but Gram-positive, tentatively place it in the genus *Corynebacterium* (the diphtheroids) of Lehmann and Neumann.

The intracellular parasites are transmitted from generation to generation through the eggs of every individual. The follicular cells of the ovary become infected. Young oöcytes are free from infection, but the more mature ova show a number of bacilli at their surfaces. As the ova grow, the bacteria at their surfaces become more numerous and form a closed layer around the periphery. Still later a definite congregation occurs at each pole. The penetration of the eggs by the parasites occurs late, since the bacteria first actually appear within the eggs after oviposition.

The bacteria are seen in ovarian sections or in films prepared from ova excised from mature females. They are also found within the developing embryos when these are removed from their egg capsules. The parasites found congregated along the periphery of older ova and within developing eggs and embryos are illustrated in Fig. 2. They are slightly more pleomorphic than the bacteriocyte individuals. Short bacilli and coccoidal forms are more numerous than the longer bacilli. The cocci are apparently derived from buds pinched off from longer rods. It appears that the division rate of the parasites is accelerated within the eggs and embryos. In this material the rods have a length range of 0.8 to 4.8  $\mu$ , with a mean length of 0.9  $\mu$ .

### 3. Method Used to Obtain Tissues Free from Contaminants

We have found the following method satisfactory:

The roach is etherized until immobile. It is then submerged for 15 to 20 minutes in a solution consisting of equal parts of 95 per cent alcohol and 1/1000 mercuric chloride, after which the insect is washed in 70 per cent alcohol. The extremities are amputated at their bases with sterile scissors and the insect is fastened with sterile pins ventral side up in a tray of paraffin recently melted and hardened. Prior to using the paraffin, the tray is flooded with 70 per cent alcohol for a few minutes. After pinning, the ventral side of the abdomen is again washed off with 70 per cent alcohol, and by means of a fine pipette alcohol is forced in between the external abdominal segments. The abdomen is lastly singed with a red hot spatula, and the ventral chitin removed with sterile instru-

ments. Fat or ovarian tissue is then removed from the body cavity and transferred to sterile tubes. Before the tissues are used for cultures, they are macerated with the addition of sterile water. The alimentary tract and any of its adnexa must be avoided.

The female of *Periplaneta americana* lays a hard chitinous egg capsule partitioned in the interior and containing anywhere from 20 to 24 eggs or embryos. Since the eggs and embryos harbor "symbionts" or "rickettsiae," it appeared possible to sterilize the exterior of the capsule, open it, remove the eggs or embryos, macerate them and inoculate media with this material. Before this was accomplished, however, a preliminary microscopic and cultural study seemed necessary. We had no means of knowing whether the interior of the capsule or the partitioned spaces were sterile. Furthermore, the eggs are surrounded with a mucilaginous secretion which might not be sterile. Consequently, freshly deposited capsules were opened and the mucilaginous material examined in the stained state. No microorganisms were revealed. Broth, nutrient agar and dextrose horse blood agar slants and plates were inoculated with the mucilaginous secretion, but subsequently on incubation remained sterile. Macerated eggs and embryos, however, revealed the characteristic diphtheroids. It seems safe to conclude, therefore, that the interior of the normal roach egg capsule is sterile for ordinary bacteria. Normal appearing egg capsules, laid while the insects are in the wild state, are unsafe to use, because some of them are diseased. It is safer to keep captive female roaches under observation during the process of oviposition and take the capsules as soon as they are free.

Capsules so obtained were submerged in the corrosive sublimate-alcohol mixture, previously described, for 30 minutes. They were then washed for 15 minutes in 70 per cent alcohol and rinsed in sterile physiological saline solution. The exteriors of the capsules were tested for sterility by placing them for 4 days in nutrient bouillon and incubating. If the sterilization proved effective the capsules were opened in a small amount of sterile physiological salt solution, the eggs removed and crushed, and the media inoculated.

#### 4. Isolation Experiments

Attempts were at first confined to transplanting macerated *Periplaneta* ova and bacteriocytes to media at various hydrogen ion concentrations under both aerobic and anaerobic conditions. The results were usually negative. In a modified Noguchi's medium, a suspicious diphtheroidal form was initiated three times and twice an adaptation from this medium to standard nutrient agar was obtained.

*Medium and Methods Adopted.*—The medium and methods finally adopted are as follows:

Standard nutrient agar (10-12 cc.) is poured into petri dishes into which previously 3 cc. of sterile defibrinated horse blood and 2 cc. of a 10 per cent sterile dextrose solution has been added. The final pH value of this medium should be adjusted to a range of from 7.0-7.4. The plates are incubated for 48 hours to test their sterility for ordinary contaminants.

A standard platinum loopful of the macerated ovarian, embryonic or bacteriocyte material, obtained under sterile conditions, is taken and the surface of the medium touched and gently rubbed in an area having a diameter of approximately 0.5 cm. What remains on the loop is then carried over to another area which is gently rubbed in a similar manner. This "spotting technique," as the writer has named it, may be continued without refilling the loop, so that a petri dish may contain anywhere from 2 to 15 or more spots. The number of spots made is entirely arbitrary. After the "spotting," the plates are incubated for 48 hours at 35 or 36°C. They are then examined and it is seen that the spots are dry. If all of the directions are followed very few or no contaminating colonies will develop. When such colonies do appear they are more likely to occur around the edges of the plate and in areas not inoculated. The spots usually appear entirely sterile. Fat drops and other material introduced may persist and give the appearance of minute pinpoint colonies, but actual colonies representing developing microorganisms are difficult to detect. Fishings from the spots are made, nevertheless, and transferred to slants of the same medium as that used in the petri plates. It is important to have a sufficient amount of water of condensation present in the slants. If the quantity appears deficient, a few drops of a solution consisting of 5 cc. bouillon and 1 cc. horse blood serum should be added. In making fishings a loopful of the condensation fluid from a slant is carried over to a spot. The drop is rubbed around on the spot and carried back to the slant and streaked. This procedure is repeated for each spot, so that one finally obtains as many streaked slants as spots present on a plate. The slants are then incubated at 36°C. or are held at room temperature. The tubes should be examined every day after 48 hours and tilted to permit the water of condensation to flood the surface of the medium. A growth may appear anywhere from 48 to 144 hours. At the end of this time, if sterile, the tubes may be discarded. If a growth occurs it is extremely faint and delicate consisting of minute, clear or pale white colonies of pinpoint size.

*Results with Protocols.*—Many unsuccessful attempts were made to obtain primary cultures on slants. Transfers were always prepared from these although at the time no visible growth appeared. However, after the initial development on the plates occurs the microorganisms are easily adapted to the blood agar slants and later to certain other media.



In the first experiment (Table I), two plates represented material from one egg capsule.

Plate 1 was "spotted" 9 times; Plate 2, 6 times. When more than one plate was used the loop was always recharged with the suspension. The plates were incubated 48 hours. At the end of this time, each plate contained two contaminating bacterial colonies, not in close proximity to any of the spots. Fish-

TABLE I  
*Isolation Experiment*  
*Egg Capsule*

From Plate I Slant No.	36°C. 24 hrs.	36°C. 48 hrs.	Room T. 72 hrs.	Room T. 96 hrs.	Room T. 120 hrs.	Room T. 144 hrs.	Type
1	Sterile						
2							
3	Sterile	+	+	+	+	+	II
4	Sterile						
5							
6							
7							
8							
9							
From Plate II Slant No.							
1	Sterile	+	+	+	+	+	II
2	Sterile						
3							
4							
5							
6							

\* + signifies growth.

ings were made from all the spots, so that 9 streaked slants represented Plate 1, and 6 Plate 2. These slants were held for 2 days at incubator temperature and for the remainder of the time, up to 144 hours, at room temperature. Slant 3, from Spot 3, Plate 1, yielded a delicate growth in 48 hours. Slant 1, from Spot 1, Plate 2, yielded a similar growth in the same time. These cultures were transferred indefinitely on dextrose blood agar slants and were subsequently classified as Type II. The other "fishings" remained sterile and were discarded.

Table II represents an experiment with another egg capsule.

Two plates were "spotted" and 9 spots fished from one, 6 from the other. Plate 1 remained sterile for visible colonies; Plate 2 yielded two contaminants, not near any of the spots. On Slant 1, from Spot 1, Plate 1, a delicate growth was obtained in 144 hours. This growth was subsequently classified as Type III. The remaining tubes proved sterile and were discarded.

TABLE II  
*Isolation Experiment*  
*Egg Capsule*

From Plate I Slant No.	36°C. 24 hrs.	36°C. 48 hrs.	Room T. 72 hrs.	Room T. 96 hrs.	Room T. 120 hrs.	Room T. 144 hrs.	Type
1	Sterile	Sterile	Sterile	Sterile	Sterile	+	III
2	Sterile						
3							
4							
5							
6							
7							
8							
9							
From Plate II Slant No.							
1	Sterile						
2							
3							
4							
5							
6							

Table III represents an experiment performed with fat tissue bacteriocytes and with ova dissected from mature ovaries.

One plate was "spotted" with each and two control plates accompanied the series. These controls were "spotted" with sterile water, incubated, and later "fished." Material from each control spot was transferred to dextrose horse blood agar slants and incubated as in the experiments. Control plates were prepared for all the remaining experiments and all slants from them remained sterile for diphtheroids. Plate 1, from the ova, yielded 4 contaminating colonies.

One contaminant was found on the edge of one spot, but was avoided during the fishing process. Plate 2, from the fat tissue, yielded 2 contaminating colonies, not on any of the spots. Two contaminants, not on spots, developed on each

TABLE III  
*Isolation Experiment*  
*Fat Tissue and Ova from One Female Roach*

From Plate I ova Slant No.	36°C. 24 hrs.	36°C. 48 hrs.	36°C. 72 hrs.	36°C. 96 hrs.	36°C. 120 hrs.	36°C. 144 hrs.	Type	Control plate I
1	Sterile	Sterile	Sterile	Sterile	+	+	III	Sterile
2	"	"	"	"	+	+	II	
3 4	Sterile							
5	Sterile	Sterile	Sterile	+	+	+	II	
6 7 8	Sterile							
9	Sterile	Sterile	Sterile	Sterile	Sterile	+	III	
10 11	Sterile							
From Plate II fat tissue Slant No.								Control plate II
1	Sterile							Sterile
2	Sterile	Sterile	Sterile	+	+	+	I	
3 4	Sterile							
5	Sterile	Sterile	Sterile	Sterile	+	+	I	
6	"	"	"	"	+	+	II	

control plate. Slants from the ovarian material yielded Types II and III twice each; slants from the fat tissue bacteriocytes, Type I twice and Type II once. The remaining slants remained sterile.

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Table IV represents the result obtained on slants from a small number of fishings from ova and fat tissue from two female roaches.

The fat tissue bacteriocytes yielded Type II, on Slant 2, from Spot 2, Plate 3, Female 1. No contaminating colonies developed on Plate 1 from Roach 1. Plate 2, from the same roach, gave two contaminants not on any of the spots.

TABLE IV  
*Isolation Experiment*  
*Fat Tissue and Ova from Two Female Roaches*

Roach I Plate I ova Slant No.	36°C. 24 hrs.	36°C. 48 hrs.	36°C. 72 hrs.	36°C. 96 hrs.	36°C. 120 hrs.	36°C. 144 hrs.	Type	Control plate I					
1	Sterile						II	Sterile					
2													
3													
4													
Roach I Plate II ova Slant No.													
1													
2													
3													
4													
Roach I Plate III fat Slant No.								Control plate II					
1								Sterile					
2	Sterile	Sterile	+	+	+	+							
3													
Roach II Plate I fat Slant No.													
1													
2													
3													

Two contaminants, not on the spots, developed on Plate 3 from Roach 1. One contaminant, not on a spot, developed on Plate 1 from Roach 2. No contaminants developed on the two control plates.

An experiment, not tabulated, was performed with fat tissue bacteriocytes from a female roach.

The fishings from the two plates remained sterile. A contaminating colony, not on a spot, developed on each experimental plate; two developed on control Plate 1 and one on control Plate 2.

Table V illustrates the results obtained with fat and ovarian tissue from two females and fat tissue from a male.

TABLE V

*Isolation Experiment*

*Fat Tissue and Ova from Two Female Roaches; Fat Tissue from One Male Roach*

Roach I Plate I ova Slant No.	36°C. 24 hrs.	36°C. 48 hrs.	36°C. 72 hrs.	36°C. 96 hrs.	36°C. 120 hrs.	36°C. 144 hrs.	Type	Control plate I
1	Sterile	Sterile	Sterile	Sterile	Sterile	Sterile		
2	"	+	+	+	+	+	III	Sterile
Roach I Plate II ♀ fat Slant No.								Control plate II
1 2	Sterile							Sterile
Roach II Plate I ♀ fat Slant No.								Control plate III
1 2 3	Sterile							Sterile
Roach III Plate I ♂ fat Slant No.								Control Plate IV
1	Sterile	Sterile	Sterile	+	+	+	I	
2 3	Sterile							Sterile

Plate 1, ovarian material from Roach 1, remained sterile for contaminating colonies. Slant 2 from Spot 2, yielded Type III, however. Two contaminants, not on spots, developed on control Plate 1. Plate 2, fat material from female Roach 1, remained sterile for diphtheroids. One contaminating colony developed in one of the two spots, but was avoided in fishing. Another contaminant was noticed along the edge of the plate. One contaminant developed along the edge of control Plate 2. Plate 1, fat material from female Roach 2, remained sterile

for diphtheroids. Two contaminating colonies developed but were avoided. One was noticed along the periphery of a spot, the other beyond any of the three spots. No contaminants developed on control Plate 3. Plate 1, fat material from male Roach 3, yielded Type I diphtheroid on Slant 1 from Spot 1. Two contaminating colonies, not diphtheroids, developed along the periphery of Spots 2 and 3. These were avoided in fishing and the slants from these two spots remained sterile. No contaminants developed on control Plate 4.

Table VI represents the result obtained with ovarian material from a female roach.

TABLE VI  
*Isolation Experiment*  
*Ova from a Female Roach*

Plate I Slant No.	36°C. 24 hrs.	36°C. 48 hrs.	36°C. 72 hrs.	36°C. 96 hrs.	36°C. 120 hrs.	36°C. 144 hrs.	Type	Control plate
1	Sterile	Sterile	+	+	+	+	I	Sterile
2	Sterile							
3								
4								
5								
6	Sterile	Sterile	Sterile	Fungus contam- ination	Dis- carded	Dis- carded		
7	Sterile							
8								
9								
10								
11								
12								
13								
14								
15								

The fishing from Spot 1 yielded Type I. The rest remained sterile, with the exception of Slant 6 upon the surface of which a fungus developed. Three contaminants grew upon the experimental plate. These were found removed from any of the fifteen spots. Two contaminants grew along the edge of the medium on the control plate.

An experiment, not tabulated, resulted negatively.

One contaminating colony developed in a spot and three others elsewhere. No contaminants developed on the control plate.

Table VII gives the result with fat tissue bacteriocytes from a female roach.

No contaminating colonies developed, but two, beyond the spots, appeared on the control plate. Type II diphtheroid developed on Slant 1 from Spot 1.

TABLE VII  
*Isolation Experiment*  
*Fat Tissue from a Female Roach*

Plate I Slant No.	36°C. 24 hrs.	36°C. 48 hrs.	36°C. 72 hrs.	36°C. 96 hrs.	36°C. 120 hrs.	36°C. 144 hrs.	Type	Control plate
1	Sterile	Sterile	+	+	+	+	II	Sterile
2	Sterile							
3								
4								
5								
6								
7								
8								
9								
10								
11								

TABLE VIII  
*Isolation Experiment*  
*Fat Tissue from a Male Roach*

Plate I Slant No.	36°C. 24 hrs.	36°C. 48 hrs.	36°C. 72 hrs.	36°C. 96 hrs.	36°C. 120 hrs.	36°C. 144 hrs.	Type	Control plate	
1	Sterile							Sterile	
2									
3	Sterile	Sterile	Sterile	+	+	+	II		
4	Sterile								
5									
6									
7									
8									
9									
10									

Table VIII shows that Type II on Slant 3 from Spot 3 was the only significant result.

Three contaminants were obtained on the experimental plate but they grew outside of the spots. Two contaminants, outside of the spots, appeared on the control plate.

The experiment given in Table IX yielded Type I on Slants 3 and 11, and Type III on Slants 9 and 10.

Three contaminating colonies beyond the spots, developed on each plate, both in the experiment and in the control.

TABLE IX  
*Isolation Experiment*  
*Fat Tissue from a Female Roach*

Plate I Slant No.	36°C. 24 hrs.	36°C. 48 hrs.	36°C. 72 hrs.	36°C. 96 hrs.	36°C. 120 hrs.	36°C. 144 hrs.	Type	Control plate
1 2	Sterile							Sterile
3	Sterile	+	+	+	+	+	I	
4 5 6 7 8	Sterile							
9	Sterile	+	+	+	+	+	III	
10	"	+	+	+	+	+	III	
11	"	+	+	+	+	+	I	
12 13 14	Sterile							

*Analysis of the Isolation Experiments.*—Fishings were made from the spots on the plates after 48 hours although no macroscopic growth was visible. At first the incubation of the plates was prolonged 5 days, but this had no greater advantage than the 2 day incubation. Stained films direct from the spots, after 24 hours, showed many degenerating and Gram-negative diphtheroids. A few remained normal and retained the gentian violet. These few Gram-positive rods seemed physiologically active and were in the process of adaptation



to their new environment. The first conditions appeared necessary to initiate this adaptation but were not sufficient to bring its realization to complete fruition. When transplanted to the second environment (dextrose horse blood agar slants) further development proceeded and small colonies were formed. Further adaptation resulted during successive transplants. Altogether 138 spots were made and 20 cultures obtained from these. Approximately 14 per cent of the attempts succeeded.

The time consumed between the plate "fishings" and the appearance of a growth on the slants varied. Seven cultures were macroscopically observed in 48 hours, three in 72 hours, four in 96 hours, four in 120 hours, and two in 144 hours. No further cultures were obtained after 144 hours.

The first few spots on the plates, in general, yielded a greater percentage of slant cultures than the last. This was probably due to progressive dilution. The fact remains that six cultures were obtained from the first spots, four from the second, three from the third, two from the fifth, one from the sixth, two from the ninth, one from the tenth, and one from the eleventh.

In regard to the three types, Types I and III were each recovered 6 times and Type II, 8 times. The three types were recovered from females, Types I and II from males, and Types II and III from capsules. This does not signify that Type III does not occur in males, nor Type I in capsules. The data on these points are too small. One individual usually yielded only one type, but in one case the three types were recovered from one female, and Types I and III were recovered from another. Types II and III were recovered from ova and embryos, and Types I and II from mature fat tissue bacteriocytes. The number of times each type was recovered from the ova is practically identical with the frequency with which the same type was recovered from the mature bacteriocytes.

The few contaminations encountered on many of the experimental and control plates were derived from the atmosphere during the hardening of the media. After pouring the plate, the cover was permitted to remain partly open for a few minutes in order to allow the water vapor to escape, as is customarily done. This procedure may have been the principal source of the contaminants, but they usually developed at a considerable distance from where the material was planted

and were avoided during the fishing process. Moreover it is significant that no control plate yielded diphtheroidal microorganisms.

Many unsuccessful attempts were made to isolate diphtheroidal microorganisms from the alimentary tract, from the feces and from the exterior of *Periplaneta*. Recently, however, one spot from one plate out of four inoculated with a fecal emulsion yielded a form resembling Type III. The intracellular parasites undoubtedly often gain an entrance into the digestive system due to the omnivorous and filthy feeding habits of roaches. The many unsuccessful attempts to isolate the microorganism from this source, however, seem to show that few remain viable after ingestion.

### 5. Morphological, Cultural, and Biological Characters of the Three Types

The three types cultivated from *Periplaneta* are Gram-positive, not acid-fast, not motile, non-sporulating and non-capsulating. All have a tendency to clump. When 48 hour cultures on dextrose horse blood again are fixed while wet in alcohol-ether for 15 minutes and stained

TABLE X

*Measurements of the Periplaneta Parasites, Compared with Those of the Three Cultured Types*

Source of parasites	Length range	Mean length	Breadth range	Diameter range of coccoidal forms
	$\mu$	$\mu$	$\mu$	$\mu$
Roach bacteriocytes.....	2-5	3	0.5-1	0.5-1
Roach ova.....	0.8-4.8	0.9	0.5-1	0.5-1
Culture Type I.....	0.5-3.2	1.95	0.4-0.6	0.5-1
Culture Type II.....	1.6-4.8	3.49	0.5-1	0.5-1
Culture Type III.....	1.6-3.2	2.12	0.4-0.6	0.3-0.8

according to Giemsa, they show the following characters illustrated in the figures. Type I, Fig. 3, is a moderately thin banded or bipolar bacillus with a few oval or coccoidal forms. The bacilli are straight, slightly curved or crescent-shaped. Type II, Fig. 4, is a longer and thicker banded bacillus showing racket or club-shaped terminations. These bacilli are also usually flexed and at times form chains of two or three units. Oval and coccoidal forms are also present and appear to be derived from terminal buds formed by the bacilli. Equal and unequal division appears to occur. Type III, Fig. 5, represents an

extremely pleomorphic type, a coccobacillus or a rickettsia form. Banded straight or slightly curved bacilli are present but the oval and especially the coccoidal forms dominate. By comparing the cultural forms with the forms obtained directly from *Periplaneta* (Figs. 1 and 2), differences are seen but these are fundamentally slight. The morphological characters of cultures are the most reliable ones which can be used for differentiating the three types.

Differentiation of the three types on a cultural basis is not possible. However, slight differences as they consistently appeared, immediately after isolation, will be given. Growth on the best medium, dextrose horse blood agar, is weak, but by successive transfers the microörganism was gradually adapted to certain routine media. Practically all of the cultural characters reached their peak in 120 hours at 36°C.

In standard nutrient bouillon, no clouding appeared up to 8 days. A slight, viscid sediment was formed at that time. Microscopical examination in 120 hours demonstrated a weak growth of the three types, with a tendency to agglutination. Nutrient bouillon containing 20 per cent horse serum and 1 per cent dextrose showed moderate clouding in 120 hours for Type I. Types II and III did not cloud this medium although a microscopical examination revealed growth. Moderate growth was obtained for all three types on Dorsett's egg medium. Milk seemed a poor medium; no coagulation was obtained in one month, although at that time the milk had a slightly acid reaction. Potato yielded a poor growth. Nutrient agar slants after 120 hours showed a weak growth consisting of a few clear, round, minute, pinpoint colonies. Coagulated horse serum slants yielded a slightly heavier growth of the same character as on the nutrient agar. Dextrose horse blood agar slants showed a good, although delicate growth in 120 hours. In 8 days the pinpoint colonies became pale white in color. Horse blood agar slants, without dextrose, yielded a weaker growth. Gelatin stabs held at 22°C. in 8 days showed a very weak growth, beaded along the line of puncture. In 2 months no liquefaction occurred. Dextrose horse blood agar plates gave clear, round or irregularly round, pinpoint colonies in 120 hours which later assumed a pale white color. For Type I these colonies measured  $1/3$  to  $1/2$  mm. in diameter. For Types II and III the colonies measured up to  $3/4$  mm. in diameter reaching 1 mm. in diameter in 8 days. Types I and II colonies also possessed a slightly raised center. Growth was inhibited on nutrient agar plates. On 1 per cent dextrose agar plates a few colonies appeared which had the same appearance as those on dextrose horse blood agar.

Carbohydrate fermentation tests did not yield any differential characters. The three types do not produce any gas; they ferment glucose, sucrose and maltose, but not lactose or mannite. Since horse serum stimulated the growth of the microorganisms slightly, 1 per cent of each carbohydrate was added to

nutrient bouillon containing 20 per cent of horse serum. Before adding the maltose to the serum bouillon, the latter was heated at 60°C. for 30 minutes (7).

The maximum acidity, reached in some cases in 5 days, in others not before 17 days, is recorded in Table XI.

TABLE XI

Carbohydrates.....	Glucose	Lactose	Sucrose	Maltose	Mannite
pH of medium .....	7.3	7.3	7.3	7.3	7.3
" " Type I .....	5.2	7.3	5.2	5.3	7.4
" " " II .....	5.2	7.3	5.0	5.3	7.4
" " " III .....	5.4	7.2	5.6	5.3	7.3

Agglutination tests were undertaken to determine, if possible, a further qualitative or quantitative difference between the three types. Some diphtheroids do not form antibodies readily, so a prolonged and varied treatment of rabbits is often necessary.

Rabbits at first received 13 intraperitoneal inoculations, at suitable intervals, of 1.5 cc. of living microorganisms derived from cultures gradually adapted to nutrient agar slants and upon the surface of which good, but delicate 120 hour growths were obtained. The entire 120 hour growth from a slant was suspended in 2 cc. of physiological salt solution and used for each inoculation. Three intraperitoneal inoculations with 2 cc. followed. The rabbits were then bled from their ears, but the titer was still low. Three intraperitoneal inoculations again followed, given on three consecutive days, 0.5 cc. on the 1st, 1 cc. on the 2nd, and 2 cc. on the 3rd day. The rabbits were then rested for 10 days, after which they received intravenous inoculations on 3 consecutive days, 1 cc. on the 1st, 1.5 cc. on the 2nd, and 2 cc. on the 3rd. One week following this procedure blood serum was again obtained.

Table XII gives the tests performed with suspensions of nutrient agar cultures 120 hours old of the three types against normal and immune rabbit sera. Due to the tendency to natural clumping which the three types exhibit, all suspensions were thoroughly shaken prior to performing the tests.

A marked distinction exists between the three types. Spontaneous complete agglutination at all dilutions occurred with Type II and normal serum as well as with salt solution, but since this never occurred with the other two types it must be accepted as a difference.

Table XIII shows a series of agglutination tests with the three types against their own immune sera and against the immune sera of



TABLE XIII  
Reciprocal Agglutination Tests

R. W. GLASER

TABLE XIII

Reciprocal Agglutination Tests

	I	II	III																			
Immune serum.....	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480	1/40960	1/81920								
Serum dilutions.....	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480	1/40960	1/81920								
Type I.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
" II.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
" III.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
Immune serum.....	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480	1/40960	1/81920								
Serum dilutions.....	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480	1/40960	1/81920								
Type I.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
" II.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
" III.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
Immune serum.....	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480	1/40960	1/81920								
Serum dilutions.....	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/20480	1/40960	1/81920								
Type I.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
" II.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
" III.....	+	+	+	+	+	+	+	+	+	+	+	+	+	+								

the others. Marked differences are noticeable with a tendency for Type II to agglutinate strongly at all dilutions and with each of the sera.

### 6. Filtration Experiment

Filtrable stages of certain rickettsiae have been reported in the literature. Since the three types cultivated by us are rather pleomorphic, especially Type III, it was important to determine whether stages exist small enough to pass the pores of diatomaceous filters.

Dextrose blood agar slant cultures, 120 hours old, of the three types were separately suspended in 10 cc. physiological salt solution. These solutions were filtered through Berkefeld grade "N" candles at a pressure of 74 mm. of mercury. The time of filtration and the amount of the filtrates obtained were constant. Each filtrate was then centrifuged for 30 minutes at 1,000 R.P.M., after which 0.1 cc. of the upper liquid and 0.1 cc. of the lower portion were added to the surface of dextrose blood agar slants. All of the tubes so inoculated remained sterile after an incubation of 2 weeks, showing that nothing passed the filters.

### 7. Classification

The sizes and shapes of the three types of microorganisms studied correspond to those of the intracellular parasites of *Periplaneta americana*. The intracellular and cultivated forms are straight or curved rods with a tendency to club and racket shapes. All are pleomorphic and show barred, uneven staining. They are not acid-fast, but Gram-positive and non-motile. The cultured forms are aerobic, and since insect tissue is primarily aerobic (8), it may be assumed that the intracellular forms are adapted to oxygen. No endospores occur in the parasitic or cultured forms. These considerations, primarily, automatically place the species within the genus *Corynebacterium* (the diphtheroids). For the species we would like to propose the name *periplanetae*, and the variety studied by us may be known as *americana*, including three types. The term *Corynebacterium periplanetae* n. sp. variety *americana* has the following advantages: The generic name places the microorganism among the diphtheroidal bacilli. The specific name associates the species with the insect genus *periplaneta* and the variety name with the insect species *americana* and not with another species, as for example *Periplaneta australasiae*. Should it be later shown that all members of the genus *periplaneta* harbor the

identical species of intracellular parasite the variety name could be discarded.

8. *General Immunity of Periplaneta towards Its Intracellular Inhabitants and towards the Cultured Types*

To prove further the identity of the cultured species with the intracellular parasite, we attempted agglutination tests with the immune rabbit sera against the diphtheroids obtained from the insect fat tissue and ova. It was extremely difficult, however, to obtain heavy enough suspensions of the microorganisms from these sources. Extraneous material, such as tissue fragments, fat, etc., interfered with the reactions and with the reading of the data. Complement fixation tests were tried, but the technical difficulties which the material presented were too great and these methods were abandoned. Another approach, however, itself. The speculation which led to the experiment follows.

Roaches must exhibit a general immunity towards their intracellular parasites; otherwise these would not be restricted to certain prescribed cells, but would grow uninhibited anywhere in the body. If the cultured and intracellular forms were inoculated into the body cavity of normal roaches they should be inhibited or destroyed provided the host is sensitized to both; in other words, provided both microorganisms are members of the same species. On the other hand, bacteria foreign to the body of the roaches as a rule kill them when they gain access to the body cavity (9). Some experiments support the above speculations.

Two mature roaches received intraperitoneal inoculations of 0.1 cc. of a 10 cc. physiological salt solution suspension of a 24 hour agar culture of a *Bacillus coli* from calves; two the same dose of *Staphylococcus pyogenes albus*, two the same dose of *Staphylococcus muscae* (10), and two a culture of a bacillus isolated by the writer from *Stomoxys calcitrans*. Death of all these roaches occurred between 24 and 72 hours after infection. Cultures and films from blood and tissues showed many of the inoculated bacteria. Following this test six roaches were inoculated intraperitoneally with 0.2 cc. of 1 cc. physiological salt solution suspension of the intracellular parasites obtained from fat cell bacteriocytes. None were present in the blood 24 hours after inoculation, and the six roaches remained alive. Four hours after inoculation blood films stained with Wright's stain



revealed a few degenerated pink stained diphtheroids. The same result was obtained by inoculating each of the three cultivated organisms intraperitoneally into six roaches.

The above experiments indicate that *Periplaneta americana* is sensitized to both the parasitic and cultured forms, but is not so sensitized to other bacteria.

## 9. SUMMARY AND CONCLUSIONS

In *Periplaneta americana*, the large American roach, bacteriocytes are found in both sexes. These bacteriocytes are scattered throughout the fat tissue and their cytoplasm is filled with microorganisms. Evidence is presented to show that the intracellular forms are diphtheroidal bacilli. These diphtheroids are transmitted from one generation to another through the ova.

A method is described whereby tissues, free from contaminants, may be obtained from *Periplaneta*. A medium and a new method, the "spotting technique," are described, by means of which initial cultures of the parasites were obtained from eggs within capsules, from ova and fat tissue bacteriocytes. Two conditions appear necessary, one to initiate adaptation to the new environment, the other to bring its complete realization to fruition. When development has been properly initiated, further adaptation occurs with successive transfers until finally certain other media appear suitable. Approximately 14 per cent of the isolation and cultivation attempts succeeded. All of the isolations were studied and it was found that three morphologically distinct types had been cultivated. In general one host yielded only one type, but one female revealed three types and another two. One type was also isolated once from a fecal emulsion. The probable reason for this result is discussed.

The three types isolated were diphtheroidal bacilli resembling one another closely enough to be considered a single species but invariably offering distinct minor differences to warrant a separation into three distinct types. These three types have remained true to their original forms and sizes through 52 transfers. The sizes, general morphology, and tinctorial reactions of the three types cultivated correspond to

the intracellular parasites of *Periplaneta americana*. The cultural and biochemical activities of the strains did not reveal any sound characters for differentiating types. Serologically, however, useful distinctions were found.

Some additional evidence along immunological lines is offered to show that the microorganism cultivated is a representative of the identical species parasitic within *Periplaneta americana*.

The evidence appears sound to the writer that the *Periplaneta* parasite has been isolated and cultivated over 20 times and that it is a bacterium belonging to the genus *Corynebacterium* (the diphtheroids). For the species the name *Corynebacterium periplanetae* nov. sp. variety *americana* is proposed.

The three cultural types did not produce forms small enough to pass through the pores of Berkefeld "N" candles.

The writer wishes to acknowledge the interest and able assistance given by Mr. N. A. Coria during the course of this work.

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## EXPLANATION OF PLATE 4

Sketches drawn at 1800 diameter magnification. Alcohol-ether fixation. Stained according to the method of Giemsa.

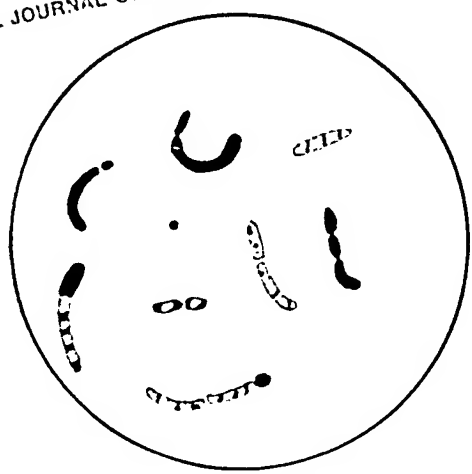
FIG. 1. *Corynebacterium periplanetae* var. *americana* from fat tissue bacteriocytes of *Periplaneta americana*.

FIG. 2. The same parasite from the ova of *Periplaneta*.

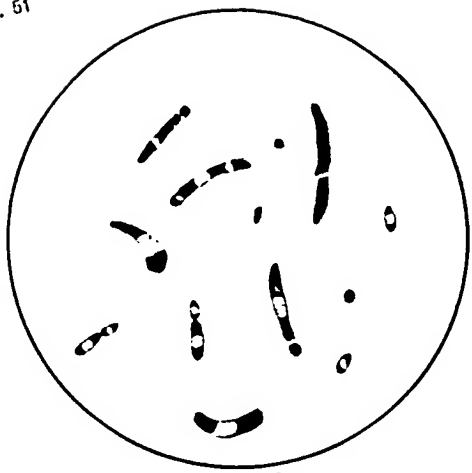
FIG. 3. Type I. 48 hour culture of the same microorganism on dextrose blood agar.

FIG. 4. Type II. 48 hour culture of the same microorganism on same medium.

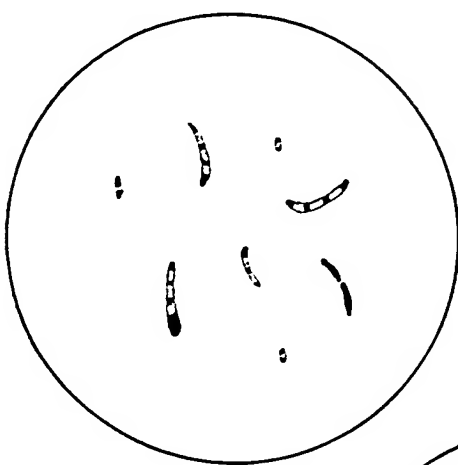
FIG. 5. Type III. 48 hour culture of same microorganism on same medium.



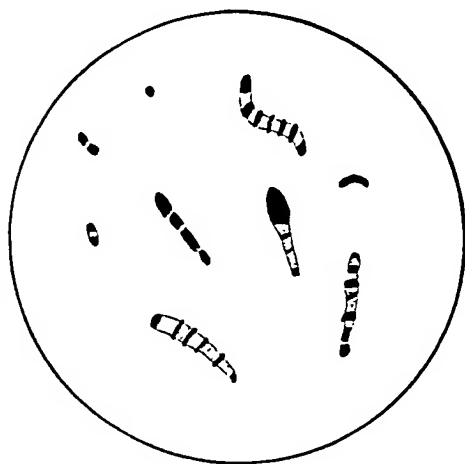
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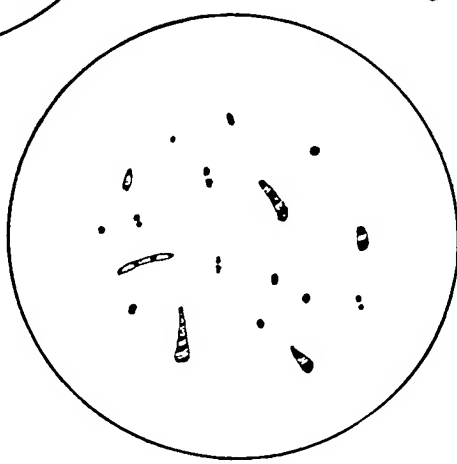
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3



4



5

R. W. G.

(Glaser: "Rickettsia" of *Periplaneta americana*)



# STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

## I. BLOOD COUNTS IN NORMAL RABBITS

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(Received for publication, October 14, 1929)

The cells of the peripheral blood in rabbits have been studied in this laboratory in connection with the problem of the constitutional factors related to the occurrence and course of disease, and with especial reference to the influence of environmental conditions (1). Although many investigations have dealt directly or indirectly with the cytology of rabbit blood comparatively little has been published regarding the blood cell picture of normal rabbits from the standpoint of a large material. Furthermore, the possibility of spontaneous variations over periods of time with respect to general environmental conditions such as seasonal change has received but scant attention. These features are among those included in the present study. Observations have also been made on various types of animal material and in connection with studies primarily concerned with factors of an environmental (experimental) nature. Still other observations have been carried out on rabbits inoculated with *Treponema pallidum*, a malignant tumor, and virus III, the first two agents being those with which most of our studies on constitutional and environmental factors have been conducted.

The results of the investigation will be published in a series of papers of which this is the first. It contains the results of a statistical analysis of 1110 blood counts obtained from 174 normal rabbits, with especial attention directed to distribution frequencies. The values obtained from this analysis will be used in subsequent papers as a basis with which to compare the results of other experiments. Preliminary notes on certain phases of the study have already been published (2).

### *Materials and Methods*

The rabbits employed were, as far as could be determined, a fair sample of the animal material available for experimental purposes. For the most part, the ordinary brown and gray types predominated but the type described as the Flemish cross or mixture was also represented, and there were a few black and albino animals. Only male rabbits approximately 6 to 8 months old were used; a few were thought to be slightly younger and a few were probably a month or two older. Each rabbit was caged separately in a well lighted (sunlight), well ventilated room; the diet throughout the period of observation consisted of hay, oats, and cabbage.

The results reported are based upon 1110 blood counts on 174 rabbits during 13 months, from October 20, 1927 to November 22, 1928. The majority of examinations, 1001 in number, were made during the period ending June 20, 1928. The observations were derived from 3 sources of animal material:

I. 10 rabbits; 426 observations. Between October 20, 1927 and June 20, 1928, blood counts on 5 animals were made at weekly intervals. With the other 5 rabbits, biweekly examinations were carried out from October 20, 1927 to February 10, 1928 and thereafter to June 19, 1928 at weekly intervals.

II. 10 rabbits; 130 observations. Weekly counts were made from March 29, to June 19, 1928.

III. 154 rabbits; 554 observations. This mixed group contains, first, single observations on 9 groups of 5 to 10 rabbits each, a total of 75 animals, examined from December 16, 1927 to August 9, 1928, and within a few days from the time of receipt from the dealers. Second, there are 470 observations distributed among 11 groups of 5 to 10 rabbits each, a total of 70 animals; these groups were examined 4 to 11 times during 1 to 8 weeks. Third, a single observation on 9 of the 10 rabbits comprising group I made 4 months after the regular examinations had been discontinued, is included in this group.

A uniform routine with respect to the time and the method of collection and examination of the blood was followed. The rabbits were fed as usual on the day preceding the examination but received no food on that day until after the specimens of blood had been obtained. The majority of observations were made from 9 A.M. to 1 P.M. In the case of 2 groups with a large number of observations, the interval at which examinations were made was largely determined by other experiments in progress; the rabbits were always examined in the same order on the same day of the week and at the same or approximately the same hour.

The blood was obtained from an ear vein without disturbance by simply placing the rabbit upon a rough surface such as a towel stretched tightly across a table top. An assistant supported the head and held the ear forward from its base in a convenient position. The external surface of the ear was moistened with dilute alcohol and a small area was carefully shaved. The shaved skin was dried with gauze and the vessels dilated by means of an electric bulb beneath the ear. A small vessel was punctured with the point of a corneal knife and a few drops of blood allowed to flow freely before any was collected. The specimens for the following

examinations were then obtained in the order designated: the differential white count (supravital technic), the white cell count, the hemoglobin estimation, and the red cell count. All specimens from 4 rabbits were taken one after the other and the counts were then made immediately. The method of Sabin (3) was followed for the supravital preparations, vital neutral red dye<sup>1</sup> being used and 100 white cells in each specimen counted. Standardized pipettes and the usual dilutions of 1:100 of Hayem's solution for the red cells and 1:10 of a 1.0 per cent acetic acid solution for the white cells were employed. The hemoglobin content was determined by the Newcomer hemoglobinometer, using 0.2 cc. blood in 10 cc. of  $\frac{N}{10}$  hydrochloric acid.

In the analysis of results, absolute numbers of cells per cmm. of blood have been considered. The distribution curves given in Text-figures 1 to 8 are based upon the use of a group interval of approximately one-tenth of the mean value of each class of cells. The curves obtained by plotting the actual results were smoothed by the formula  $\frac{a + 2b + c}{4}$ ; the initial value of each smoothed curve is represented by  $\frac{2b + c}{3}$  and the final value by  $\frac{a + 2b}{3}$ . In some instances, the curves are not extended to the upper limits but the values omitted are few and scattered. The limit to which a curve should be extended is indicated by a figure placed at the termination of the curve.

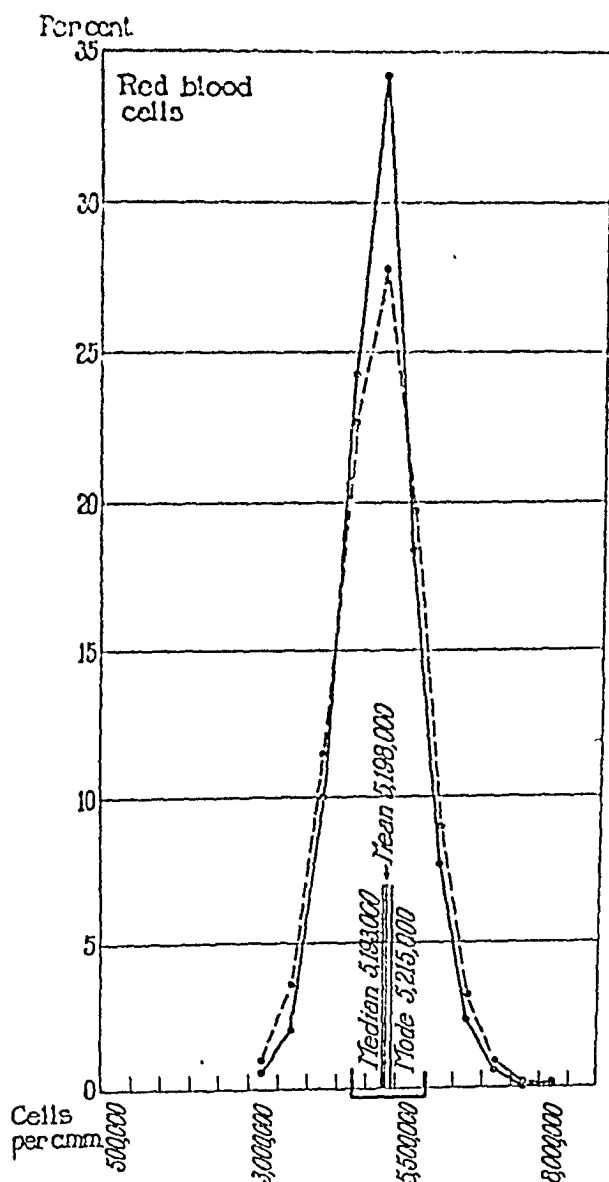
The mode as reported was obtained by the use of King's formula applied to the unsmoothed distribution curve,  $\text{mode} = 1 + \frac{f_2}{f_2 + f_1} c$ , in which 1 is the lower limit of the group in which the greatest number of values is massed,  $f_2$  the frequency in the group above, and  $f_1$  the frequency in the group below the modal group, and  $c$  the size of the group interval. By varying  $f_1$  and  $f_2$  to represent the combined frequencies of the 2 or 3 groups below and above the modal group, the formula was in reality applied to a smoothed curve. In the case of the hemoglobin in which the modal group is approached by an adjoining group,  $c$  represents 2 groups. The empirical formula of Pearson,  $\text{mode} = \text{mean} - 3(\text{mean} - \text{median})$ , and the mean of the modal group were also used to check the results of the above method.

The figures as given include all data. No count has been omitted because of the occurrence of such conditions as snuffles or ear canker in occasional rabbits observed for the longer periods. In all other cases, the animals were apparently free from disease. It seemed best to present the material in the present form be-

<sup>1</sup> The dye employed was neutral red iodide No. 2 supplied through the kindness of Dr. Barnett Cohen, Hygienic Laboratory, Washington, D. C.



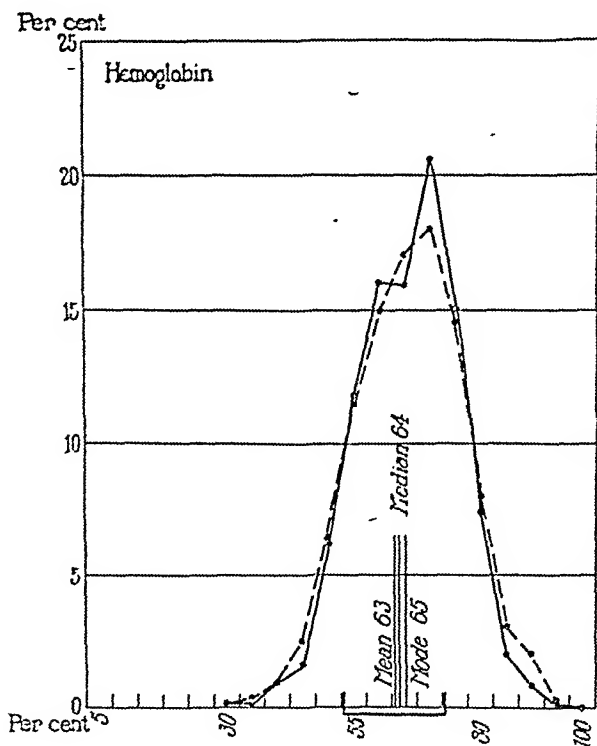
fore attempting to make corrections for disease or any other factor that might be associated with variations in the numbers of cells.



TEXT-FIG. 1. Distribution frequency of numbers of red blood cells per cmm. in per cent. In this and subsequent text-figures, the smoothed curve is graphed as a broken line and the brackets on the base line denote the limits of the standard deviation.

## RESULTS

The results obtained in this series of 1110 blood cell determinations on 174 normal rabbits are presented in condensed form in Table I and Text-figures 1 to 8. The analysis of this material has included the determination of the following numerical values for the total red and white cells and the several classes of white cells per cmm., and the hemoglobin content: minimum, maximum, and mean counts, the probable error of the mean, the mode, the median, the standard deviation and the coefficient of variation (Table I). The distribution frequencies of the cells and the hemoglobin have also been determined (Text-figs. 1 to 8).

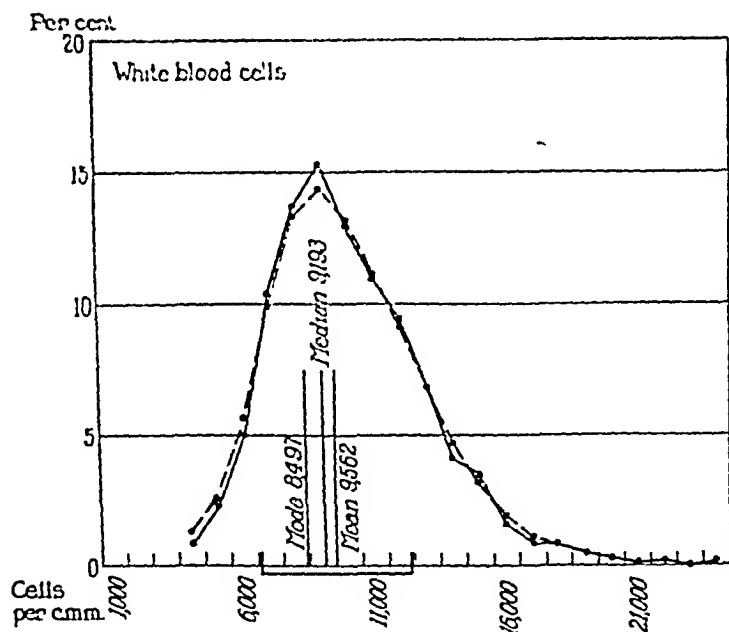


TEXT-FIG. 2. Distribution frequency of percentage of hemoglobin content in per cent.

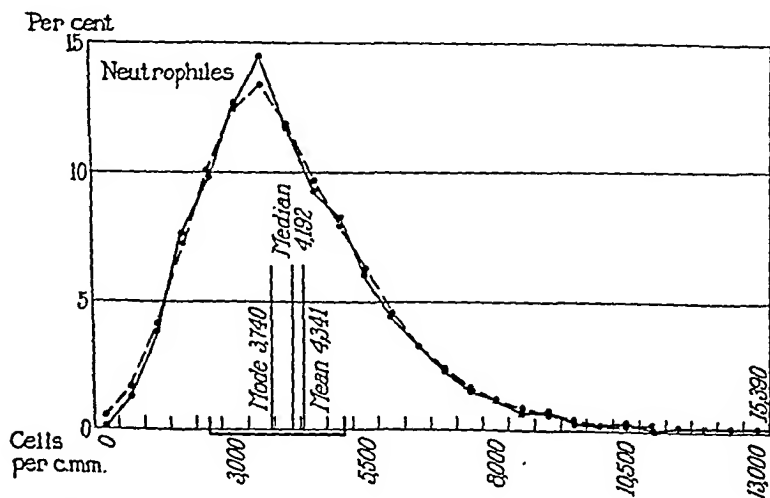
TABLE I

*Summary of Numerical Values Obtained from 1110 Blood Counts on 174 Male Rabbits of Various Ages and Breeds—from October 20, 1927 to November 21, 1928*

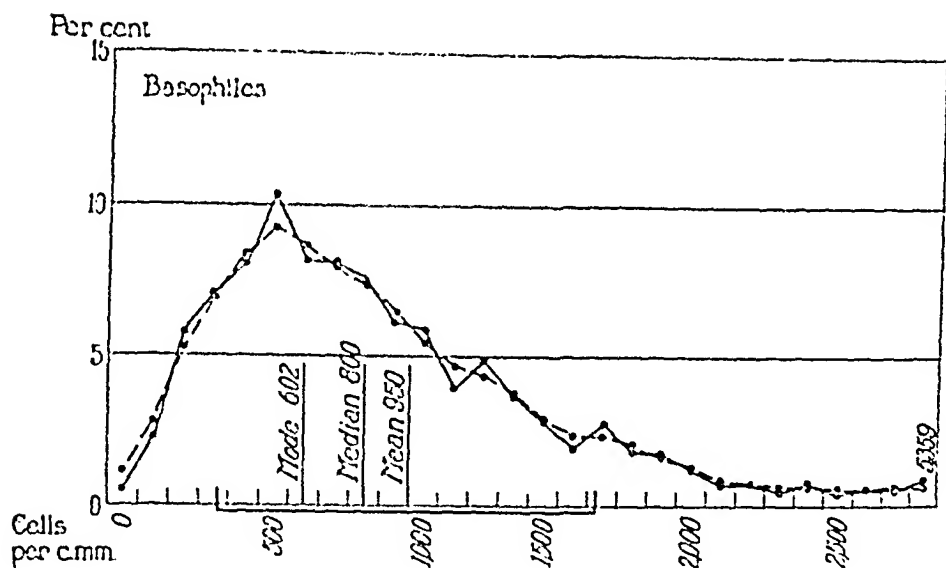
	Mean	Mode	Median	Minimum	Maximum	Standard deviation	Coefficient of variation
	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cmm.</i>	<i>per cent</i>
Red blood cells.....	5,198,000 $\pm$ 12,700	5,215,000	5,193,000	3,020,000	8,040,000	628,250	12.09
Hemoglobin.....	63% $\pm$ 2%	65%	64%	28%	90%	10%	15.37
White blood cells.....	9562 $\pm$ 59	8497	9193	3150	23500	2919	30.53
Neutrophiles.....	4341 $\pm$ 37	3741	4192	1050	15390	1823	41.99
Basophiles.....	950 $\pm$ 13	602	800	0	5359	635	66.84
Eosinophiles.....	214 $\pm$ 4	90	159	0	1760	217	101.40
Lymphocytes.....	3045 $\pm$ 28	2402	2904	630	9900	1366	44.86
Monocytes.....	1000 $\pm$ 12	750	898	72	5405	571	57.10



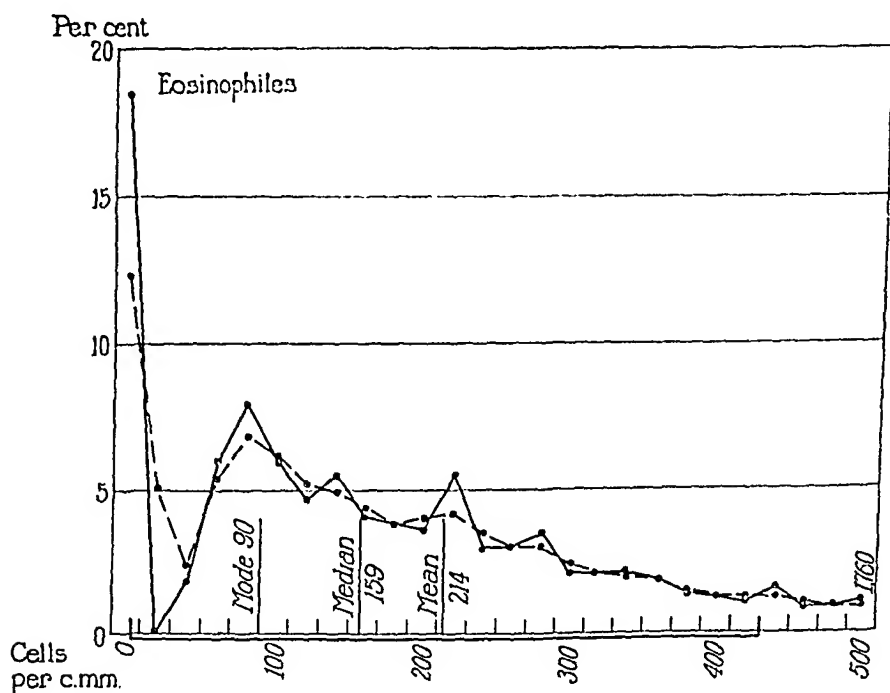
TEXT-FIG. 3. Distribution frequency of total numbers of white blood cells per cmm. in per cent.



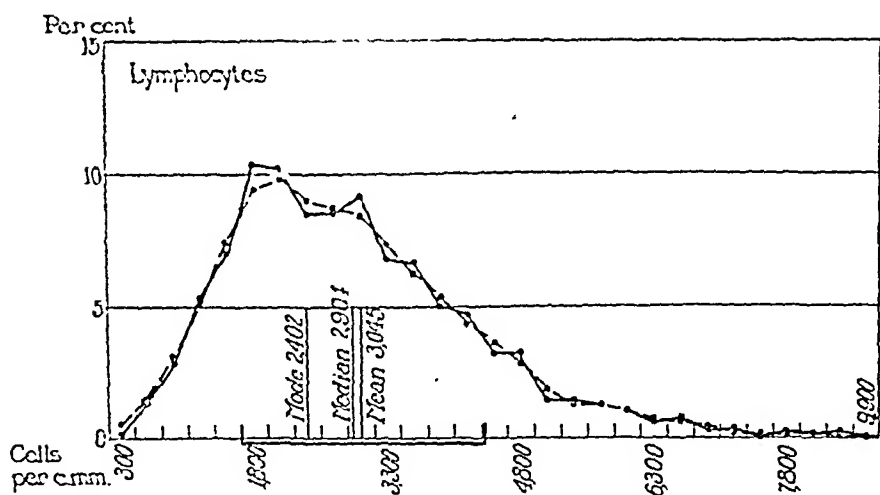
TEXT-FIG. 4. Distribution frequency of numbers of neutrophils per cmm. in per cent.



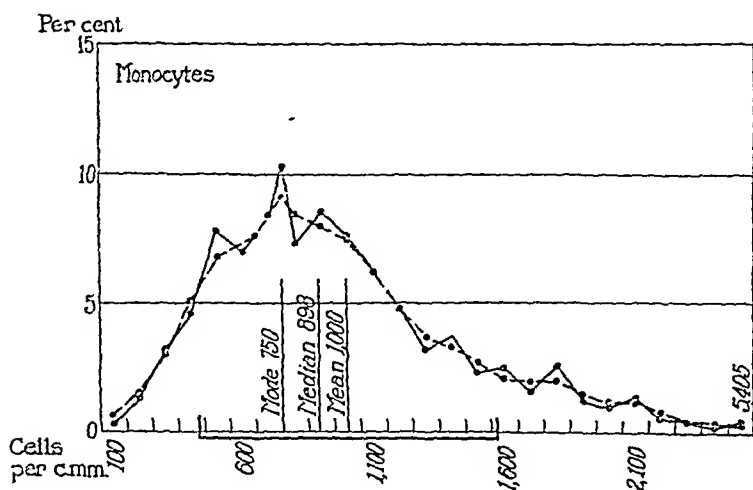
TEXT-FIG. 5. Distribution frequency of numbers of basophiles per cmm. in per cent.



TEXT-FIG. 6. Distribution frequency of numbers of eosinophiles per cmm. in per cent.



TEXT-FIG. 7. Distribution frequency of numbers of lymphocytes per cmm. in per cent.



TEXT-FIG. 8. Distribution frequency of numbers of monocytes per cmm. in per cent.

## DISCUSSION AND CONCLUSIONS

In discussing the results of these experiments, it should be borne in mind that no effort was made to maintain uniformity of material other than that the rabbits employed were comparable with those used in other experiments. And the same may be said regarding the conditions of living while the animals were under observation. Various breeds of rabbits were used, certain groups were studied for short and others for long periods of time, while other series were examined but once. These conditions, of course, tend to emphasize the element of variability. One purpose of this study, however, was to ascertain the possible as well as the probable limits of variation in the numbers of cells and in the hemoglobin content. In the present paper, the results are considered primarily from the standpoint of the range of individual counts rather than from that of the various groups of rabbits comprising the entire series.

An examination of the data contained in Table I and in the distribution curves of Text-figs. 1 to 8 shows that with one exception, the numbers of cells in the peripheral blood of rabbits are subject to wide variations. The exception is the erythrocytes, the coefficient of variation of which is 12.09 per cent, a value much lower than those of the various granulocytes, the lymphocytes, and the monocytes. As might be expected in view of this finding, the variation of the hemoglobin content is of a similar order of magnitude, its coefficient of variation being 15.87 per cent. In contrast to these findings, the various white cells are characterized by a high order of variability. The coefficients of variation of the neutrophilic leucocytes and the lymphocytes are 41.99 and 44.86 per cent; those of the basophiles and monocytes are somewhat higher, 66.84 and 57.1 per cent while that of the eosinophiles reaches the high value of 101.4 per cent. The variability of the total white blood cells, on the other hand, occupies a lower level, its coefficient of variation being 30.53 per cent.

It will be seen that the distribution curves for the red blood cells (Text-fig. 1) and the hemoglobin content (Text-fig. 2) have the same general form and are almost symmetrical; they show a relatively narrow range of numerical distribution with a rapid rate of increase and decrease in the percentage of counts whose numbers are close to the mean and mode and have but a slight tendency to prolongation at either

extreme. Both curves, however, show a slight left skew, a feature which is brought out more clearly by the relative positions of the mean, the median, and the mode. The differences between the values for the mean and mode are extremely small, 17,000 cells for the erythrocytes and 2 per cent for the hemoglobin respectively. It is not unlikely that with a larger number of observations the curves would conform to theoretical expectations. In the case of the hemoglobin, the curve in its ascending portion at the 60-65 per cent level shows a peculiar flattening which is reflected in the more rounded shape of the ascending as compared with the descending limb of the smoothed curve. The explanation of this peculiar feature is not entirely clear but it may be a question of the matching of shades at this particular level in making the readings.

The distribution frequencies for the other cells are much more irregular. With the single exception of the eosinophiles (Text-fig. 6), the total white cells, the neutrophiles, the basophiles, the lymphocytes, and the monocytes (Text-figs. 3, 4, 5, 7, 8) have a tendency to follow the same general form of numerical distribution as the red blood cells and the hemoglobin content but the curves are of a much wider range and are decidedly skewed to the right, due to the occurrence of varying counts at levels well above the mean. The form of the curves for the total white cells and the neutrophiles shows that the most frequent values occupy a considerably narrower range than those of the basophiles, lymphocytes, and monocytes. In the case of these 3 classes of cells, the highest portion of the curves shows a fairly wide plateau, the right extreme of which corresponds approximately to the level of the mean while the left falls below the mode. The relative positions of the mode, the median, and the mean conform to theoretical expectations in all cases.

The curves for the total white blood cells and the neutrophiles are quite regular but those of the other classes of white cells show many irregularities chiefly in their descending portions. These conditions extend the zone of high frequency distribution and in addition, are responsible for the high coefficients of variation. The distribution curve for the eosinophiles (Text-fig. 6) differs from all the others with respect to the high level of its initiation, but if this portion of the curve is omitted, its general form resembles the others. It is, however,



somewhat flatter. The peculiar first part of the curve is due to the fact that in many counts no eosinophiles were seen. In order to make the chance of including a fair representation of eosinophiles equal to that of the monocytes and basophiles for example, it would be necessary to count 500 instead of 100 cells upon the basis of the ratio of the respective mean values for these cells which is approximately 5:1 (Table I). As with the other classes of white cells, the curve for the eosinophiles shows a marked skew to the right indicating a wide range of high frequencies. Its irregularities (descending portion), however, are more pronounced than those of the other cells with the possible exception of the monocytes. These conditions make for the high order of magnitude—101.4 per cent—of the coefficient of variation.

The curves for the total white cells and the neutrophiles are very similar and at first glance, it might be supposed that the distribution of neutrophile values was responsible for this similarity. But the neutrophiles represent approximately only half the total number of white cells (Table I) and since the coefficient of variation of the total white cells is less than that of the neutrophiles, it is evident that the other cells, considered collectively, would form a distribution curve not unlike that of the neutrophiles and with a smaller coefficient of variation than those of the individual classes of cells. This would suggest that while these various classes of cells vary widely, they tend to preserve some kind of a relation so that their total numbers, from a collective point of view, are approximately as constant as the numbers of neutrophiles.

The results of this study with respect to the mean values for the red and white blood cells and for the neutrophiles are of the same general order as those reported by others, as for example Bushnell and Bangs (4), Scott and Simon (5), and Cunningham, Sabin, Sugiyama, and Kindwall (6) (Table II). With the other classes of white cells, however, there is less agreement except in the case of the last named authors whose mean values for the lymphocytes and monocytes differ but little from those here reported. It should be noted that the differential counts of Bushnell and Bangs, and presumably those of Scott and Simon, were made from fixed preparations while Cunningham, Sabin, Sugiyama, and Kindwall used the supravital technic.

TABLE II  
*Comparison of Blood Cell Values as Given by Various Authors*

	Number of counts	Number of rabbits
Bushnell and Bangs.....	100	100
Scott and Simon.....	100	100
Cunningham et al.....	217*	53
Pearce and Casey.....	1110	174

	Mean	Standard deviation	Coefficient of variation	Percentage of white blood cells
	<i>per cmm</i>	<i>per cmm.</i>	<i>per cent</i>	<i>per cent</i>
Red blood cells				
Bushnell and Bangs.....	5,989,500	779,358	13.01	
Pearce and Casey.....	5,198,000	628,000	12.09	
White blood cells				
Bushnell and Bangs.....	10,675	2224	20.85	
Scott and Simon.....	11,105			
Cunningham et al.....	11,281			
Pearce and Casey.....	9,562	2919	30.53	
Neutrophiles				
Bushnell and Bangs.....	4174	1161	27.57	39.1
Scott and Simon.....	3825			34.4
Pearce and Casey.....	4341	1823	41.99	45.4
Basophiles				
Bushnell and Bangs.....	382	232	60.61	3.4
Scott and Simon.....	135			1.2
Pearce and Casey.....	950	635	66.84	9.9
Eosinophiles				
Bushnell and Bangs.....	120	90	75.00	1.1
Scott and Simon.....	374			3.3
Pearce and Casey.....	214	217	101.40	2.2
Lymphocytes				
Bushnell and Bangs.....	5754†	1196	20.77	53.9
Scott and Simon.....	6297			56.7
Cunningham et al.....	2805			24.9
Pearce and Casey.....	3045	1366	44.86	31.8
Large mononuclears				
Bushnell and Bangs.....	49	54	118.60	0.43
Scott and Simon.....	532			4.7
Transitionals				
Bushnell and Bangs.....	114	96	84.11	1.07
Monocytes				
Cunningham et al.....	943			8.43
Pearce and Casey.....	1000	571	57.1	10.5

\* This value which does not appear in their publication, was supplied by the authors.

† Small lymphocytes only.

In regard to the coefficient of variation, the values of Bushnell and Bangs and those reported here are in close agreement for the red blood cells but with the other cells the differences range from 6.23 in the case of the basophiles to 26.4 for the eosinophiles. Similar differences are also found in the standard deviation values of the two series. In view of the experimental and technical circumstances, however, it is not surprising that the results of several series of observations, such as those contained in Table II, do not agree more closely. The relative numbers of counts and of animals employed, the method used for the differential white cell counts, and the nature of the animal material are among the factors which obviously affect the nature of results of this character.

Although the present study is based upon a large number of observations, a comparatively large number of normal stock rabbits and an acceptable method of white cell differentiation, the values submitted for the total red and white cells counts, for the numbers of the various classes of white cells, and for the hemoglobin content in the peripheral blood should be accepted as approximate values of normality. They serve the useful purpose of orientation and in addition, may be employed as a basis of comparison for the results of other experiments. But it will become evident when the various small groups comprising the series and the factors affecting them are considered, that the influence of existing conditions must be taken into account before any value be accepted as a standard of normality.

#### SUMMARY

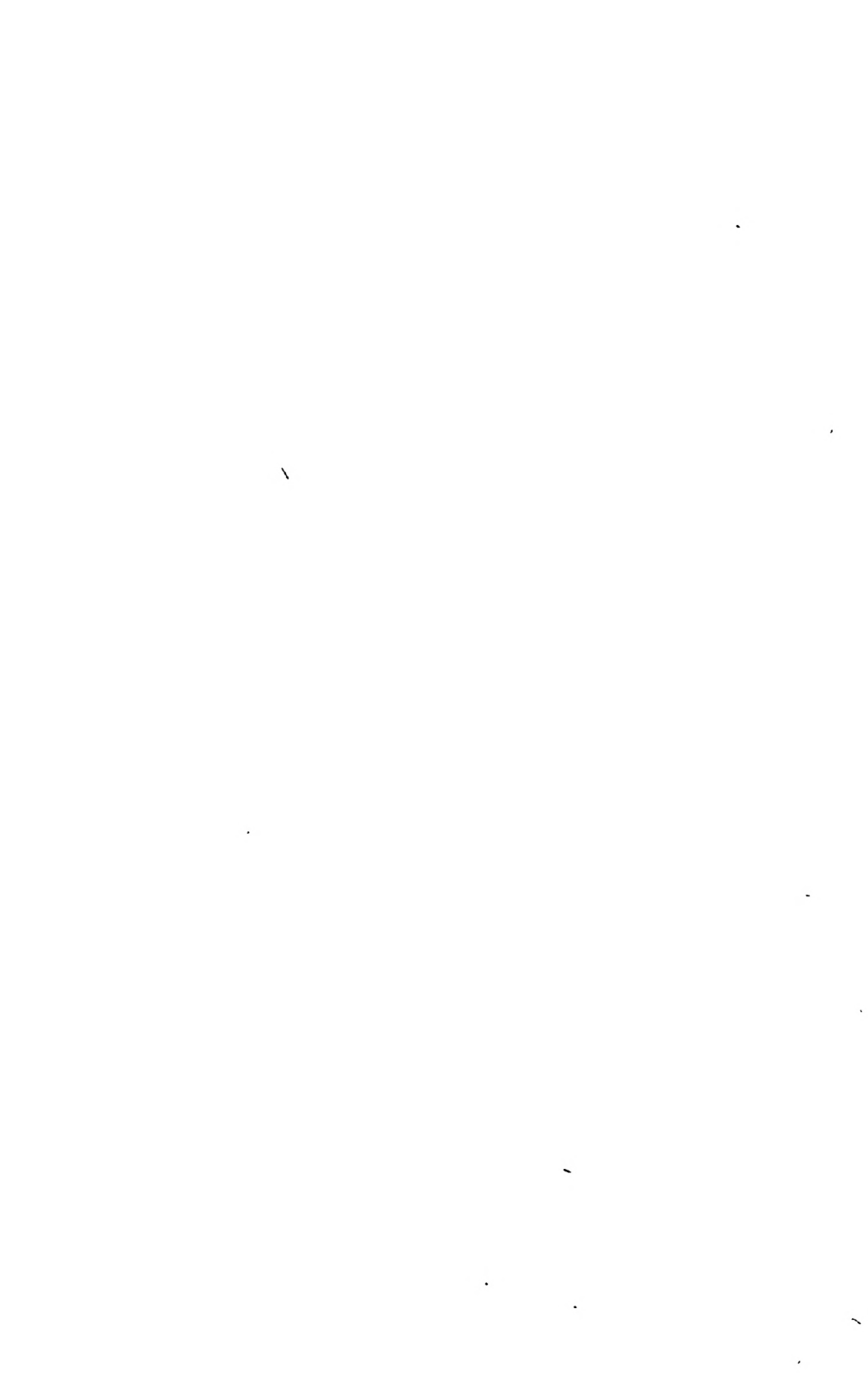
A study of the blood cytology of normal male rabbits was carried out from October 20, 1927 to November 22, 1928 in connection with an investigation of constitutional and environmental factors related to the occurrence and course of disease. In 1110 observations on 174 animals, total red and white cells counts, differential white cell counts by the supravital method, and hemoglobin estimations were made.

A statistical analysis of the results obtained is presented. Attention is directed to the occurrence of wide variations in the numbers of the various white cells as contrasted with comparatively small variations in the numbers of red cells and of hemoglobin content.

The results recorded are regarded as representing approximate rather than fixed values of normality.

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# THE TRANSFORMATION OF PNEUMOCOCCAL TYPES

## I. THE CONVERSION OF R FORMS OF PNEUMOCOCCUS INTO S FORMS OF THE HOMOLOGOUS TYPE\*

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In previous communications (1, 2) it was shown that avirulent, non-type-specific R forms of *Pneumococcus* may be converted into virulent, type-specific, S organisms either "*in vivo*" by animal passage, or "*in vitro*" by growth in anti-R serum. Conversion by both methods was invariably accompanied by the acquisition of all the characteristics of the S type, including maximal virulence. In every instance in which the change was effected by these procedures the R forms were converted to the same specific type from which they were originally derived.

Since the publication of the foregoing results there has appeared a most significant article by F. Griffith (3), in which he stated that:

1) R forms of *Pneumococcus* may be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of large amounts of living R organisms.

2) R forms of *Pneumococcus* may be similarly converted into S forms of the homologous type by the subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of homologous S cultures.

3) R forms of *Pneumococcus* may be transformed into S forms of heterologous types by the subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of heterologous S cultures.

The present communication is concerned with the first two of the

\* In this and succeeding instances 'homologous type' indicates that specific S type from which the R forms were originally derived.

above findings. The third finding, which involves the question of actual transformation of type, is the subject of the succeeding paper.

*A. Conversion of R Forms of Pneumococcus into S Forms of the Homologous Type by the Subcutaneous Injection, in White Mice, of Large Amounts of Living R Organisms*

*Methods*

Ten-hour plain broth cultures of *Pneumococcus* were centrifuged and the bacteria were resuspended in plain broth in varying dilutions as outlined in the following experiments. Mice were inoculated subcutaneously in the right inguinal region, with 0.5 cc. of each of the various dilutions, care being taken that none of the material escaped along the track of the needle. All mice were autopsied in the following manner: The skin was washed with alcohol and a subcutaneous incision was made along the mid-line of the abdomen. A flap of skin was then reflected and cultures were made from the site of injection on blood agar plates and in blood broth. The inguinal gland, or a portion of tissue from this region, was carefully excised under sterile precautions and cultures were made from this material in blood broth. Except in earlier experiments contaminations rarely occurred. Occasionally mice developed ulcers at the site of injection and such animals were discarded. Cultures were made from the heart's blood in the usual manner.

EXPERIMENTAL

*(a) 2 R culture, strain D/39/R.*

This culture was obtained from a typical Type II S *Pneumococcus* by growth in homologous immune serum. Its virulence was such that 0.5 cc. of culture occasionally killed white mice, but amounts of 0.25 cc. or less uniformly failed to do so. It produced only Rough colonies, did not agglutinate specifically in type sera, and did not produce the specific soluble substance upon which type-specificity depends (4). It could be changed to the S type by either the "*in vivo*" method of animal passage, or by the "*in vitro*" method of growth in media containing anti-R serum. Three to four mouse passages usually sufficed to bring about the R→S change, while five to seven transfers in 10 per cent anti-R serum induced a similar transformation. Single-cell cultures derived from the mass culture have been shown to react in precisely the same manner.

A series of eight mice were injected subcutaneously in the right inguinal region with the bacteria from varying amounts of culture, a volume of 0.5 cc. being injected in each instance.

TABLE I

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
2	10	1 d. 40 hrs. 2 " " "	R and S " " "	S "	Type II " "
2	5	1 d. 18 hrs. 2 " 40 "	R only R and S	R only S	R Type II
2	2.5	1 d. 40 hrs. 2 " 72 "	" " " " " "	" "	" " " "
2	1	1 d. 30 hrs. 2 " 48 "	" " " " " "	" "	" " " "

d; died.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No. mice injected, 8.

Died, 8. Reversion to the homologous S type 7.

No reversions, only R organisms recovered, 1.

In seven out of eight animals injected, type-specific organisms, possessing all the attributes of the S type, were recovered from the heart's blood. Cultures from the site of injection yielded a mixture of R and S colonies. The one animal yielding only R forms died in 18 hours, apparently before there was time for the transformation to be effected.

(b) 3 R culture, strain M/3/R.

This culture was obtained from a typical Type III S *Pneumococcus* by growth in homologous immune serum. It possessed all the characteristics of the R form and could be converted to the S type, although not so readily as the 2 R culture above described. Twenty to thirty animal passages by the intraperitoneal route were necessary to restore type-specificity, while ten to fifteen transfers in 10 per cent anti-R serum were required to effect the R→S change.



Ten mice were injected subcutaneously as follows:—

TABLE II

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
2	30	1 d. 18 hrs. 2 " 48 "	R only R and S	R only S	R Type III
2	15	1 d. 48 hrs. 2 " 64 "	R and S " " "	S S	" " " "
2	10	1 d. 18 hrs. 2 " 100 "	R only R and S	R only S	R Type III
2	5	1 d. 40 hrs. 2 s.	R and S —	S —	" " —
2	2.5	1 s. 2 s.	— —	— —	— —

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No. mice injected, 10.

Died, 7. Reversion to the homologous S type, 5.

No reversion, only R organisms recovered, 2.

Survived. 3.

While this culture reverted to the homologous S type in five out of ten animals injected, larger doses were required than with the 2 R culture described. This finding is in accord with the results obtained by the other methods of inducing reversion.

(c) 1 R culture, strain 1/192/R.

This R culture had been under artificial cultivation in this laboratory for many years. All previous efforts to effect reversion to the S type had been unsuccessful. Reimann (5) passed the same strain through 105 consecutive mice without altering its characteristics and 100 transfers in 10 per cent anti-R serum likewise induced no change.

Nine mice were injected subcutaneously, each with the bacteria from 50 cc. of R culture, as follows:

TABLE III

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
9	50	1 d. 18 hrs.	R only	R only	R
		2 " " "	" "	" "	"
		3 " " "	" "	" "	"
		4 " " "	" "	" "	"
		5 " 21 "	" "	" "	"
		6 " " "	" "	" "	"
		7 " 60 "	" "	" "	"
		8 " 80 "	" "	" "	"
		9s.	—	—	—

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No mice injected, 9.

Died, 8. Reversion to the homologous type, O.

No reversion, only R organisms recovered 8.

Survived, 1.

In spite of the large amounts of organisms employed,—the bacteria from 50 cc. of culture—this strain, in all animals, failed to revert to the S type. Smaller amounts of culture were used in other experiments with uniformly negative results. This finding offers further evidence of the existence of different degrees of constancy in the R form of *Pneumococcus* and confirms the results previously obtained.

### *Conversion of Single-Cell R Cultures into S Forms of the Homologous Type*

In a previous paper (2) it was reported that single-cell cultures have always reacted in the same manner as the mass cultures from which they were obtained. To substantiate this finding single-cell R strains were selected from the above mass cultures, according to the method of Avery and Leland (6), and injected subcutaneously into white mice. In all cases essentially the same results were obtained as when the

mass cultures were used. The following protocol shows the results of a typical experiment:

*Single-cell strain, 2 R culture, strain D/39/R.*

TABLE IV

No mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
2	10	1 d. 24 hrs. 2 " 40 "	R only R and S	R only S	R Type II
2	5	1 d. 22 hrs. 2 " 24 "	R only " "	R only " "	R R
2	2.5	1 d. 54 hrs. 2 s.	R and S —	S —	Type II —
2	1	1 d. 40 hrs. 2 s.	R and S —	S —	Type II —

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No. mice injected, 8.

Died, 6. Reversion to the homologous S type, 3.

No reversion, only R organisms recovered, 3.

Survived, 2.

In this experiment three mice apparently died prematurely of an R infection. Otherwise the results were essentially the same as those recorded in Table I in which the mass culture was employed.

#### *Attempts to Cause Further "Degradation" of an R Culture by Prolonged Growth in Homologous Immune Serum*

In the course of later work it became essential to have a 2 R culture which would not revert so readily to the S type. Accordingly the above 2 R strain was grown in 50 per cent Type II serum for twelve further transfers and the resulting culture injected into mice as follows:—

TABLE V

No. mice	Amount of living R forms injected—in terms of original culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
			P.I.	H.B.	
2	10	1 d. 21 hrs. 2 " 40 "	R only R and S	R only S	R Type II
2	5	1 d. 40 hrs. 2 " " "	R and S " " "	S "	Type II " "
2	2.5	1 d. 54 hrs. 2 s.	R and S —	S —	Type II —
2	1	1 d. 70 hrs. 2 s.	R and S —	S —	Type II —

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No. mice injected, 8.

Died, 6. Reversion to the homologous S type, 5.

No reversion, only R organisms recovered, 1.

Survived, 2.

By comparison with the results in Table IV it is seen that, in spite of twelve further transfers in 50 per cent homologous immune serum, this strain reverted to the homologous S type as readily as the original culture.

It would appear that an R culture possesses the capacity to become "stabilized" at some phase of the "degradation" process. In subsequent experiments evidence will be offered to show that a corresponding condition of partial degradation may occur in S cultures. In other words, the terms R and S, as applied to cultures of *Pneumococcus*, have only a relative value.

The results of the preceding experiments may be summarized as follows:—R forms of *Pneumococcus* can be converted into S forms of the homologous type by the subcutaneous injection in white mice, of suitable amounts of R cultures. As in other methods, conversion was

invariably accompanied by the acquisition of all the attributes of the S type, including maximal virulence. Single-cell cultures reacted in the same manner as the mass cultures from which they were derived. Attempts to cause a further "degradation" of R forms by continued growth in homologous immune serum were unsuccessful.

*B. Conversion of R Forms of Pneumococci into S Forms of the Homologous Type by the Subcutaneous Injection, in White Mice, of Small Amounts of Living R Organisms Together with the Heat-Killed Bacteria from Large Amounts of the Homologous "S" Culture*

*Methods*

It is of paramount importance to consider in detail the methods employed in the production of the vaccines<sup>1</sup> and the controls adopted to eliminate the possibility of the persistence of viable forms in the suspensions of heat-killed organisms. All vaccines were made from 1500 cc. of plain broth cultures grown in 3 liter flasks. In earlier experiments little attention was paid to the phase of growth at which the culture was killed, or to the amount of autolysis which might have taken place at the time of heating. In later experiments, however, these factors were found to be of considerable importance, and ten-hour plain broth cultures were uniformly used. Moderately heavy growth was found to be essential. After ten hours growth at 37°C. the flasks of culture were subjected to a *preliminary* heating at 60° for 10 minutes. This preliminary heating inhibited further autolysis and was found to have considerable influence on the efficacy of the vaccine. The cultures were concentrated by centrifuging and taken up in 1/100th of their original volume of plain broth. This concentrated material was transferred to glass ampules, sealed in a blow flame, and heated when totally immersed in water. The vaccines were subjected to definite temperatures for varying periods, as will later be described. Since the factors of time and heat materially altered the results obtained they were most carefully regulated. Fifteen minutes was the shortest period and 60°C. the lowest temperature to which the bacterial suspensions were exposed, and invariably this minimal exposure was found sufficient to kill all pneumococci. In many experiments, however, the vaccines were heated at much higher temperatures and for longer periods of time.

Mice were injected subcutaneously in the right inguinal region, the total volume introduced never exceeding 0.75 cc. Under these conditions the development of ulcers at the site of injection did not occur except in occasional instances. The

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<sup>1</sup> For the sake of convenience the term vaccine is used to denote a suspension of heat-killed organisms.

animals were autopsied as described in the first part of this paper. Contaminations were rarely encountered. Cultures were invariably made, both from the site of injection and the heart's blood, on blood agar plates and in blood broth. The colonies were examined under a Zeiss "plate culture" microscope; but morphology alone was never considered a final criterion as to the nature of the organisms constituting the colony. Agglutination tests were done on all cultures. In cases of doubt a second mouse was inoculated and the organisms from the peritoneal contents were typed in the usual manner.

*Controls on the Viability of the Vaccines:*

The possibility of potentially viable organisms surviving in the concentrated vaccines demanded that more than ordinary control measures should be adopted to eliminate such a contingency. The "*in vitro*" and "*in vivo*" controls employed were as follows:

*1. In Vitro Controls:*

(a) Cultures were made from all vaccines in blood broth and on blood agar plates. In many experiments this was done in varying dilutions. In no instance was growth obtained.

(b) Many lots of S vaccine were used repeatedly in "*in vitro*" attempts to secure the R→S change. Broth containing concentrated S vaccine was seeded with R forms and subcultured serially for twenty transfers. No growth of "S" organisms occurred and the final culture remained avirulent for mice.

(c) In one critical experiment cultures were made, both aerobically and anaerobically, in 5 per cent blood broth and blood-extract dextrose broth. The cultures were incubated two weeks, plates poured, and the material injected into mice. No growth occurred and the mice survived.

*2. "In Vivo" Controls:*

(a) Control mice were injected with the vaccine alone. At least four mice were always used and in many experiments the number of control animals was equal to the number of experimental animals. Varying amounts of vaccine up to and including the bacteria from 100 cc. of culture were injected. Both the subcutaneous and intraperitoneal routes were used. Without exception all animals survived. They were sacrificed at intervals up to three weeks and autopsied. The inguinal lymph gland, or a portion of subcutaneous tissue at the site of injection, was dissected out and cultures were made from this material in blood broth and on blood agar plates. In some cases this tissue was ground up and injected into other mice. Cultures were also invariably made from the heart's blood. In no instance were living pneumococci recovered.

(b) Control mice were injected with the vaccine together with other live organisms. The possibility of the existence in the vaccines of potentially viable organisms, which could not multiply by themselves, but which might, in some way, be stimulated to renewed growth by other live organisms, received careful consideration. Mice were injected with vaccines together with living cultures of *Staphylococcus*, *Streptococcus*, *B. Influenzae*, and Friedländer's bacillus. All

animals which succumbed were autopsied and all surviving animals were sacrificed at appropriate intervals and careful cultures were made. In no instance was a viable pneumococcus recovered.

As Griffith pointed out (3), in certain instances, the temperature at which the vaccines were heated exerted a definite influence on the effect which they produced. The following experiments are therefore divided into two groups, 1) those in which the vaccines were heated at 60°C, and 2) those in which the vaccines were heated at 100°C. In both groups the time of heating was fifteen minutes.

*I. R Cultures Together with Homologous S Vaccines, Heated for 15' at 60°C.*

*(a) 1 R Culture (Strain 1/192/R) + 1 S Vaccine, Heated for 15' at 60°C.*

TABLE VI

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
				P.I.	H.B.	
4 Controls	cc. 90	cc. Nil.	All survived. Sacrificed 7 days	All cultures negative		
6	90	0.25	1 d. 1½ days 2 " " " 3 " 5 " 4 " 7 " 5 s. k. 7 " 6 s. k. 7 "	R only R and S S S R only R only	R only S S S — —	R Type I " " " " — —

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No. test mice injected, 6.

Died, 4. Reversion to the homologous S type, 3.

No reversion, only R organisms recovered, 1.

Survived, 2. R organisms recovered when sacrificed, 2.

(b) 2 R Culture (Strain D/39/R) + II S Vaccine, Heated for 15' at 60°C.

TABLE VII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P. I.	H. B.	
4 Controls	cc. 90	cc. Nil.	All survived. Sacrificed 7 days	All cultures negative		
6	90	0.25	1 d. 1½ days 2 " " " 3 " 2 " 4 " " " 5 " " " 6 s. ulcer at P.I.	R and S " " " " " " " " " " " " —	S S S S S —	Type II " " " " " " " " " " —

d; died.

s; survived.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No. test mice injected, 6.

Died, 5. Reversion to the homologous S type, 5.

Survived, 1. (Ulcer).



(c) 3 R Culture (Strain M/3/R) + III S Vaccine, Heated for 15' at 60°C.

TABLE VIII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
4 Controls	90	Nil.	All survived. Sacrificed 7 days	All cultures	neg- ative	
6	90	0.25	1 d. 2 days	R and S	S	Type III
			2 " " "	" " "	"	" "
			3 " 2½ "	" " "	"	" "
			4 " 3 "	" " "	"	" "
			5 " 4 "	" " "	"	" "
			6 s. k. 11 "	R only	—	—

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No. test mice injected, 6.

Died, 5. Reversion to the homologous S type, 5.

Survived, 1. R organisms recovered when sacrificed, 1.

The results of the three preceding experiments may be summarized as follows; R forms of *Pneumococcus*, when injected subcutaneously in white mice, together with S vaccines of the homologous type, were converted, in the majority of the animals, to the specific S type from which they were originally derived. In these experiments the vaccines were heated for 15' at 60°C. All control mice survived. At the end of seven days the controls were sacrificed and cultures were made from both the site of injection and the heart's blood. Without exception all cultures were sterile. The 1 R culture (Strain 1/192/R) which had remained totally avirulent after all previous efforts to effect the R→S change, reverted to the S type in three out of six animals injected.

## II. R Cultures Together with Homologous S Vaccines, Heated for 15' at 100°C.

(a) 1 R Culture (Strain 1/192/R) + 1 S Vaccine, Heated for 15' at 100°C.

TABLE IX

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. culture
				P.L.	H.B.	
4 Controls	90	Nil.	All survived. Sacrificed 7 days	All cultures negative		
6	90	0.25	1 d. 1½ days	R only	R only	R
			2 " 2 "	R only	R only	R
			3 s. } k. 9 days	—	—	—
			4 s. }	—	—	—
			5 s. }	R only	—	—
			6 s. }	—	—	—

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No. test mice injected, 6.

Died, 2. Reversion to the homologous S type, 0.

No reversion, only R organisms recovered, 2.

Survived, 4. R organisms recovered when sacrificed, 1.

Since reversion to the S type failed to occur in any of the mice which received Type I S vaccine heated at 100°, while the change was frequently effected in those which received the vaccine heated at 60°C., this experiment was repeated. Entirely similar results were obtained. Apparently, as reported in Griffith's paper, Type I vaccine, heated at 100°C. fails to produce the change brought about by the vaccine heated at 60°C.

Attention is drawn to the fact, that, in some mice, living R forms

were found in the subcutaneous tissues nine days after injection. In other experiments they have been recovered as late as twenty days after inoculation. The ability of R forms to survive in the tissues, then, is not the only condition necessary to bring about conversion to the S type.

(b) 2 R Culture (Strain D/30/R) + II S Vaccine, Heated for 15' at 100°C.

TABLE X

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
4 Controls	90	Nil.	All survived. Sacrificed 9 days	All cultures negative		
6	90	0.25	1 d. 1½ days	R and S	S	Type II
			2 " " "	" " "	"	" "
			3 " 2 "	" " "	"	" "
			4 " " "	" " "	"	" "
			5 " " "	" " "	"	" "
			6 " " "	" " "	"	" "

d; died.

P. I. place of injection.

H. B. heart's blood.

*Summary:* No. test mice injected, 6.

Died, 6. Reversion to the homologous S type, 6.

(c) 3 R Culture (Strain M/3/R) + III S Vaccine, Heated for 15' at 100°C.

TABLE XI

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in colonies		Agglutination H.B. culture
				P.I.	H.B.	
4 Controls	90	Nil.	All survived. Sacrificed 9 days	All cultures	negative	
6	90	0.25	1 d. 1½ days	R and S	S	Type III
			2 " 2 "	" " "	S	" "
			3 " " "	" " "	S	" "
			4 " " "	" " "	S	" "
			5 s. k. 13 "	R only	—	—
			6 s. k. " "	—	—	—

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

Summary: No. test mice injected, 6.

Died, 4. Reversion to the homologous S type, 4.

Survived, 2. R organisms recovered when sacrificed, 1.

The preceding experiments show that vaccines prepared from Types II S and III S organisms are equally effective in producing reversion whether heated for 15 minutes at 60°C. or for 15 minutes at 100°C. Type I S vaccine, on the other hand, while effective in causing reversion of 1 R forms to the homologous S type when heated for 15 minutes at 60°C. did not possess this property when heated for 15 minutes at 100°C.

Two possible explanations may be advanced for the apparent differences in effect produced by Type I S vaccine heated at 60° and at 100°C. First, that property of the vaccine responsible for reversion might have been destroyed, in the case of Type I vaccine, by heating at 100°C. but not in the case of Type II and Type III vaccines similarly

treated. Second, the failure of Type I vaccine to effect reversion when heated at 100°C. might not have been due to the destruction of any property of the vaccine itself, but rather to the difficulty which had always been encountered in effecting the R→S change with this particular 1 R strain. It has been repeatedly shown that the 2 R and 3 R strains employed in these experiments can be much more readily converted to the S type. However, in view of the entirely similar results recorded by Griffith, and because of the findings to be reported in the subsequent paper, the former explanation is much the more probable.

*Attempts to Effect the R→S Change by the Injection of Living R  
Organisms Together with the Heat-Killed Bacteria from Large  
Amounts of R Cultures*

It was thought that the effect of the vaccines in producing reversion might be due to one of two causes:—First, the injection of such large amounts of heat-killed culture might overwhelm the general resistance of the animal and so allow the R forms to grow in an environment suitable for the development of S types. Second, the vaccines might act locally to protect the R forms from phagocytosis, and so enable them to survive and produce their own S substance. In either case it was thought that the vaccine of an R culture, if injected in sufficiently large amounts together with living R organisms, would similarly allow reversion to take place.

2 R Culture + 2 R Vaccine of the Same Strain Heated for 15' at 60°C. (Strain D/39/R).

TABLE XII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. cultures
				P.I.	H.B.	
2	200	0.25	1 d. 2 days 2 s. k. 11 "	R only —	R only —	R —
4	150	0.25	1 d. ½ days 2 " 1 " 3 " 2½ " 4 s. k. 11 "	R only " " " " —	R only R only (2 colonies) R only (few) —	R R R —
4	100	0.25	1 d. 1 day 2 " 1½ " 3 " " " 4 " " "	R only " " " " " "	R only " " (8 colonies) R only (6 colonies) R only	R R R R

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

3 R Culture + 3 R Vaccine of the Same Strain Heated for 15' at 60°C. (Strain M/3/R).

TABLE XIII

No. mice	Amount of culture from which heat-killed organisms were obtained	Amt. of living R culture	Result	Kinds of colonies recovered in cultures		Agglutination H.B. cultures
				P.I.	H.B.	
2	200	0.25	1 d. 1 day 2 s. k. 11 "	R only —	R only —	R —
4	150	0.25	1 } 2 } s. k. 11 days 3 } 4 }	— — — —	— — — —	— — — —
4	100	0.25	1 d. ½ " 2 " 1 " 3 } s. k. 11 " 4 }	R only ' " — —	R only R only (6 colonies) — —	R R — —

d; died.

s; survived.

k; killed.

P. I. place of injection.

H. B. heart's blood.

Summary: (Tables XII and XIII).

No. test mice injected, 20.

Died, 11. Reversion to the homologous S type, 0.

No reversion, only R organisms recovered, 11.

Survived, 9. R organisms recovered when sacrificed, 0.

R vaccines, even when heated for so short a period as 15 minutes at 60°, and in huge doses, representing the bacteria from 200 cc., 150 cc., and 100 cc. of broth culture, completely failed to cause the R cultures to revert to their own type. Similar negative results were obtained when vaccines of S Friedländer bacilli were inoculated together with the R forms of Pneumococcus. Francis (7), in this laboratory, has made similar observations while working with rabbits. He found that S vaccines were effective in causing R forms to revert to the S type;

while R vaccines and vaccines of *Staphylococcus* failed to produce the change.

*"In Vitro" Attempts to Effect the R→S Change by Growth of R Forms with S Cultures and with S Vaccines*

(1) In previous work experiments had been done to observe the effect of growing R and S cultures in symbiosis. R and S forms of the same strain were grown together for serial transfers in varying dilutions, as follows:—

	“						
Dilution of S Culture.....	$10^{-1}$	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	$10^{-7}$
Dilution of R Culture.....	$10^{-1}$	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	$10^{-7}$

0.1 cc. quantities of each of the above R and S dilutions were seeded together in blood broth and daily plates and subcultures made from the mixtures. It was obvious that in such experiments considerable variation in results would be obtained, but generally speaking, the cultures maintained the same relative proportions of R and S colonies for several transfers. Apparently the mere presence and growth of S cells in intimate association with R forms was not sufficient to cause the latter to revert to the S variety.

(2) R organisms were cultured in blood broth to which was added the bacteria from 100 cc. of homologous S culture, heated for 15 minutes at 60°C. Transfers were continued for fifteen subcultures without the appearance of S colonies. At the end of this time the virulence of the R culture was determined by mouse inoculation and found to be unchanged. Such experiments suggest that the R form is not able, under ordinary "*in vitro*" conditions, to utilize an S vaccine, as such, to build up its own polysaccharide.

(3) Moreover, under similar conditions of cultivation, when 10 per cent anti-R serum was added to the media, the change failed to occur; whereas, in the absence of the vaccine, reversion regularly took place after the appropriate number of transfers. Such a result can be explained by assuming that the vaccine, which contains, in addition to its type-specific antigen, the common group-specific protein (or R) antigen (8), absorbed the anti-R antibodies from the immune serum. In the absence of the anti-R antibodies the change failed to occur. This experiment, then, offers further proof of the rôle played by anti-R antibodies in effecting R→S reversion "*in vitro*."

(4) The possibility that the vaccine might be effective in producing reversion only in the presence of tissues under anaerobic conditions received some consideration. R forms were grown, under vaseline seal, in blood broth to which was added lymph-tissue, muscle tissue and ground up spleen, in addition to large amounts of vaccine. Subcultures were made daily for six days. No S colonies appeared at any time during the process and the cultures remained avirulent.



Thus, under the conditions employed, all attempts to produce the R  $\rightarrow$  S change *in vitro* by the use of vaccines have uniformly failed.

The possibility that the vaccines became "digested" in the subcutaneous tissues of the mice, and that the R forms were able to utilize the "digestion" products to build up their own S substance also received attention. Attempts to reproduce such "digestion" "*in vitro*" will be considered in the subsequent paper.

#### DISCUSSION

The conversion of relatively avirulent pneumococci into highly virulent organisms is obviously a matter of considerable biological and epidemiological significance. Recent observations made in this laboratory suggest that R Pneumococci are not infrequently found in the flora of the upper respiratory tract of normal individuals. Such observations indicate that this form of the organism appears not only under artificial conditions of cultivation in the test-tube but may be considered as an evidence of biological adaptation to environment on the part of the bacteria. Moreover, the avirulent R form is potentially capable of again developing into the virulent S type under favorable circumstances. The factors determining such development in the human are difficult of analysis but under experimental conditions certain observations can be made in animals.

Attention is drawn first to the "*in vitro*" method of producing the R  $\rightarrow$  S change,—the growth of R organisms in anti-R serum. In this connection the existence of anti-R antibodies in the sera of normal individuals, as shown in a previous paper (2), is believed to be a point of considerable significance, and may play a rôle in the R  $\rightarrow$  S reversion process in the human being.

Griffith's observations on the conversion "*in vivo*" of avirulent R pneumococci into virulent, type-specific, S, organisms have been completely confirmed. In attempting an analysis of the causes responsible for reversion by the technique adopted by him certain points must be considered. He suggests that the mass of culture forms a nidus in which the attenuated pneumococci are protected from the bactericidal action of the tissues. But, as he himself indicates, this can play only a small part in the reversion process. Large amounts of R vaccine, amounts larger than those of the S vaccines

employed, as well as vaccines of other organisms, should also suffice to protect the R forms from phagocytosis and from the bactericidal action of the tissues. Nevertheless, reversion has never been effected under those conditions. Moreover, the finding of living R organisms in the subcutaneous tissues as late as twenty days after injection proves that opportunity to survive is not the only condition necessary to bring about the R→S change in the animal body. Other factors must play a rôle in the reversion process. Is it possible that the S vaccine, disintegrating in the animal tissues, supplies a suitable pabulum from which the living R organisms are able to resynthesize their own specific soluble substance?

A point of considerable difficulty in such a hypothesis is the explanation of the differences in results obtained by the use of Type I S vaccine heated at 60° and at 100°C. It is necessary to assume that Type I S vaccine, when heated at the higher temperature, becomes altered in such a way that it, or its disintegration products, can no longer be utilized by the R forms. But it has been shown (4) that heating at 100° in no way alters the specific carbohydrate fraction of the Type I *Pneumococcus*. This hypothesis, therefore, is inadequate unless it can be shown that the portion of the vaccine effective in causing reversion in some fraction other than the specific soluble substance as such.

The fact that large amounts of living R cultures by themselves produce the same effect as small amounts of live R cultures when the latter are injected together with S vaccines, suggests that the causes responsible for reversion under these two conditions must be closely related. In this connection it is of importance to point out that all the R strains used in these experiments yielded traces of the specific soluble substance of their original S type. This has been shown in this laboratory by Julianelle (9) in the case of the 1 R strain which offered the greatest resistance to reversion. It is reasonable, then, to assume that large amounts of living R cultures may yield a sufficient amount of S substance, (or the closely related substance necessary for reversion) from which the R forms may resynthesize their own specific polysaccharide. And apparently, once the process is initiated, it is carried on indefinitely, so long as the environment is suitable. However, on this basis, it remains difficult to explain why large amounts

of R vaccines fail to provide the necessary material; while relatively small amounts of living R cultures are able to supply this factor. It may be that the necessary substance is not present in sufficient quantities in the R vaccines; while it is elaborated in an adequate amount by the growth of the living R forms.

The failure of "*in vitro*" attempts to effect reversion by the aid of vaccines deserves some consideration. It was not found possible to induce the R→S change by growing R forms in symbiosis with S organisms, or in media containing large amounts of S vaccines. Likewise, attempts to produce the change by growing R Pneumococci with S vaccines under partial anaerobiosis in the presence of animal tissues were unsuccessful. The failure to effect reversion by these procedures suggests that either the conditions, as provided, were not adequate, or that some factor must be provided by the animal body.

It has been suggested that the S vaccine may possess some "activating" or "co-ferment" property, which, working in conjunction with the synthesizing enzymes of the R form, enables the latter to build up its own S structure. If the effect of the S vaccine is due to such a property it apparently can exercise this function only in the presence of living tissues. Moreover, one is confronted with the difficulty of explaining how such a property becomes inactivated, in the case of Type I S vaccine, by heating at 100°C., and not in the cases of Type II S and Type III S vaccines similarly treated.

Another possibility is that the conditions created in the mouse by the injection of S vaccines may be the determining factors in inducing the R→S change. It has been pointed out previously that all the R cultures used in these experiments yielded traces of specific soluble substance of their original S type. Nevertheless the animals were able to withstand the injection of comparatively large amounts of living R organisms by themselves. The mouse must therefore possess some capacity to overcome infection by organisms producing minimal amounts of S substance. It is possible that the injection of S vaccines may destroy or inhibit this limited ability of the mouse. Under such conditions the R forms may elaborate S substance in greater quantities, and as a consequence develop into S organisms.

In this connection attention is directed to the work of Sia (10). Employing serum-leucocyte mixtures in a specially constructed apparatus,

he reported the following observation. "The presence of a small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixtures so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which ordinarily possess the power to destroy such pneumococci in relatively large numbers."

However, in any of the explanations considered, it is impossible to account for the different effect produced by Type I vaccine heated at 60°C and at 100°C. The exact causes responsible for reversion, under these experimental conditions, therefore remain unexplained. Whatever they may be, the fact remains that when R cultures are injected in large amounts by themselves, or in small amounts together with the heat-killed vaccines of S organisms, the characteristics of the R organisms are actually altered. Comparable phenomena may play a rôle of great importance in many infectious processes. A focus of infection may be a point at which relatively harmless organisms assume virulent characteristics; for the subcutaneous injection, in white mice, of large amounts of avirulent pneumococci produces conditions quite analogous to those existing in a focus of infection.

#### SUMMARY

R forms of *Pneumococcus* may be converted into S forms of the homologous Type. In addition to the methods previously reported,—(1) animal passage and (2) growth in anti-R sera,—conversion may be effected by the following procedures as employed by Griffith; (1) The subcutaneous injection, in white mice, of large amounts of living R organisms. (2) The subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of homologous S cultures. There are "varying degrees of constancy of the R variant"; but by these means it has been possible to effect conversion of all R forms selected. Attempts to cause a further "degradation" of R organisms by continued growth in homologous immune serum have been unsuccessful.

Type II S and III S vaccines are equally effective in producing conversion when heated for 15' at 60°C., or for 15' at 100°C. Type I S vaccine, however, while effective in causing conversion when heated for 15' at 60°C., apparently loses this property when heated for 15' at 100°C.

R vaccines, and vaccines of other organisms, when injected together with live R cultures, have always failed to produce conversion.

The causes responsible for conversion under these experimental conditions are discussed and the possibility of the occurrence of a similar process under natural conditions in human beings is indicated.

### CONCLUSIONS

1. R forms of *Pneumococcus* may be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of large amounts of living R organisms.

2. R forms of *Pneumococcus* may similarly be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of small amounts of living R organisms, together with the heat-killed bacteria from large amounts of S cultures.

3. By these methods Types II S and III S vaccines are equally effective in producing conversion when heated for 15' at 60°C., or for 15' at 100°C. Type I S vaccine is effective in producing conversion when heated for 15' at 60°C., but not when heated for 15' at 100°C.

4. R vaccines and the vaccines of other organisms are not effective in producing conversion.

5. All "*in vitro*" attempts to produce conversion by the use of vaccines have been unsuccessful.

6. The rôle which the phenomenon of conversion may play in infectious processes is indicated.

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# THE TRANSFORMATION OF PNEUMOCOCCAL TYPES

## II. THE INTERCONVERTIBILITY OF TYPE-SPECIFIC S PNEUMOCOCCI

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In the previous communication (1) it was shown that avirulent, non-type-specific, R forms of *Pneumococcus* could be converted into virulent, type-specific, S forms of the original type by the following procedures as employed by Griffith (2): 1) The subcutaneous injection, in white mice, of large amounts of living R forms: 2) The subcutaneous injection, in white mice, of small amounts of living R forms together with the heat-killed bacteria from large quantities of the *homologous* S culture. By these procedures the R forms were invariably converted to S forms of the same specific type as that from which they had been originally derived.

Griffith further reported (2) that R forms of *Pneumococcus*, derived from S forms of any specific type, could be converted into type-specific organisms of *heterologous* S types by the following procedure:—the subcutaneous injection, in white mice, of small amounts of living R forms together with the heat-killed bacteria from large quantities of *heterologous* S cultures. In other words, he stated that it was possible to transform S *Pneumococci* of one specific type into other specific types through the intermediate stage of the R form. The present communication is concerned with the question of such transformation of *Pneumococcal* types.

### *Methods*

The suspensions of heat-killed organisms were prepared in the same manner as described in the preceding paper (1). Similar controls were employed to eliminate the possibility of the existence of viable organisms in the vaccines.<sup>1</sup> In all critical experiments the number of control animals injected was equal to the number of

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<sup>1</sup> The term vaccine indicates a suspension of heat-killed pneumococci.

test animals. The bacteria obtained from large quantities of culture, killed by heating, were also injected into animals together with cultures of other live organisms. In addition large amounts of vaccine were injected into animals intoxicated with alcohol. In no instance were viable pneumococci recovered from the controls. Notwithstanding the conclusive nature of the controls employed, even more convincing evidence as to the improbability of the existence of any viable organisms in the heat-killed suspensions will be offered in the subsequent description of the experiments.

The R strains which were used were derived in all instances from typical, type-specific, S pneumococci by growth in homologous immune serum. The possibility of the cultures containing a mixture of R forms, derived from S forms of more than one type, was eliminated in many experiments by the use of single-cell strains. In addition, proof of the nature of the R strains was obtained by converting them to the S form by growth in media containing anti-R serum (3). Under these conditions the R forms invariably reverted to the S form of the same specific type as that from which they were originally derived.

#### EXPERIMENTAL

##### *Conversion of R Forms of Pneumococcus into S Forms of Heterologous Type by the Subcutaneous Injection, in White Mice, of Small Amounts of Living R Forms, Together with Large Amounts of Heat-Killed S Forms of Heterologous Type*

In the course of early experiments it became apparent that the selection of the R strain played an important rôle in determining the results obtained. The particular 2 R culture (strain D/39/R) which was first chosen could be readily converted to the S form of the original type by all the methods which have been described: 1) animal passage; 2) growth in media containing anti-R serum; 3) the subcutaneous injection, in white mice, of large amounts of living R forms; 4) the subcutaneous injection, in white mice, of small amounts of living R forms together with the heat killed bacteria from large amounts of the homologous S culture.

In the first experiment twelve mice were injected with 0.25 cc. of living 2 R culture together with the bacteria from 90 cc. of a Type I S culture, heated for 15' at 60°C. All the mice died after an interval of 24 to 48 hours and typical Type II S pneumococci were recovered from the heart's blood of each animal. In all cases the R forms were converted to S forms of the same type as that from which they were originally derived.

Another 2 R culture, (strain N/D/39/R), was then obtained by growing a

typical Type II S pneumococcus in its homologous immune serum. After six transfers the culture was plated. An individual colony was then selected and transferred to blood broth. This 2 R culture could likewise be converted into S forms of the original type; but greater difficulty was experienced in bringing about the change than in the case of the 2 R culture previously employed. This second 2 R culture was injected into a series of twelve mice together with aliquot portions of the Type I S vaccine used in the preceding experiment. Quite different results were obtained. Ten of the twelve animals died after an interval of 24 to 48 hours and from the heart's blood of nine of these typical Type I S pneumococci were recovered. Cultures made from the heart's blood of the tenth mouse yielded only R forms. Two mice survived.

TABLE I

*Conversion of R Forms of Pneumococcus into S Forms (1) of the Original Type (2) of the Type of the Vaccine*

Type and amount of vaccine	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
				R and S forms	No. mice
Bacteria obtained from 90 cc. Type I S culture, heated at 60°C. for 15'.	Nil.	10	All survived; sacrificed 7 days.	All cultures negative.	10
ditto.	0.25 cc. 2 R (strain D/39/R)	12	All died 1-2 days.	Type II S	12
ditto.	0.25 cc. 2 R (strain N/D/39/R)	12	10 died 1-2 days. 2 survived; sacrificed 7 days.	Type I S R only. All cultures negative.	9 1

The results obtained by injecting these two different 2 R strains, together with the same Type I S vaccine, appear in Table I.

*Abstract of Protocol.—*

2 R culture, (strain D/39/R), + I S Vaccine, heated for 15' at 60°C.

Number of mice injected—12.

Number of mice died—12.

Reversion to S forms of the original type—12.

Reversion to S forms of the same type as the vaccine—0.

2 R culture, (strain N/D/39/R), + I S Vaccine, heated for 15' at 60°C.

Number of mice injected—12.

Number of mice died—10.



Reversion to S forms of the original type—0.

Reversion to S forms of the same type as the vaccine—9.

No reversion, only R organisms recovered—1.

Number of mice survived—2.

The Type I S organisms which were recovered from the heart's blood of the second series of mice possessed all the attributes of typical Type I S pneumococci. The cultures agglutinated specifically in Type I serum; they elaborated the specific soluble substance characteristic of Type I pneumococci, and were highly virulent for white mice. Subcultures made through twenty transfers retained the same properties and the organisms showed no tendency to revert to their original S type.

Since the same Type I S vaccine was used in the two experiments the variation in results obtained must have been referable to a difference in the R cultures employed. This difference was reflected in the greater difficulty experienced in causing the second 2 R culture to revert to the S form of its original type. Apparently in order that an R culture may revert to the S form of a heterologous type it must first be reduced to a definite stage in the "degradation" process.

This experiment also offers further proof of the absence of any viable organisms in the vaccine. It is highly improbable that the Type I S organisms recovered from the second series of mice could have developed from surviving forms in the vaccine; for no such forms were recovered from either the control mice or from the first series of mice which received the same vaccine.

An interesting result was obtained when still another 2 R culture was injected into mice along with Type I S vaccine. This third 2 R culture was obtained in the usual way by growing a typical Type II S pneumococcus in its homologous immune serum. When grown in anti-R serum, or injected subcutaneously in white mice in large amounts by itself, this R culture invariably reverted to the S form of the same type as that from which it was originally derived. Eleven mice were injected subcutaneously with 0.25 cc. of living R forms together with the bacteria from 100 cc. of Type I S culture heated for 15' at 60°C. Eight out of the twelve animals died after a period of one to two days, and Type I organisms were recovered from the heart's blood. In certain respects, however, the organisms recovered were not typical S forms. Although the cultures agglutinated specifically in Type I serum and produced the specific soluble substance characteristic of Type I pneumococcus, they did not possess maximal virulence for white mice nor

did they produce typical Smooth colonies. As a rule the cultures proved fatal to white mice in dilutions of  $10^{-5}$  cc., occasionally in dilutions of  $10^{-6}$  cc., but never in dilutions of  $10^{-7}$  cc. The colonies were atypical in appearance, having neither the glossy, shiny surface of Smooth colonies nor the finely granular, ground-glass appearance of Rough colonies. For the most part the colonies were irregular in outline with nibbled margins. The outer zone was usually slightly rough; toward the center the majority presented a smooth wavy appearance; and in the middle of the colony there was frequently a crater-like depression. Other colonies more closely resembled typical R colonies and still others were suggestive of those of the S variety. That such colonies were not composed of a mixture of Rough and Smooth organisms was repeatedly proven by selecting individual colonies, subculturing, and plating for several transfers. Even when passed through mice colonies with the same characteristics were recovered from the heart's blood. These forms most probably represented incompletely developed S organisms partially "stabilized" at this phase of the reversion process. Such cultures could be converted into typical S forms by the subcutaneous injection in white mice of large amounts of the cultures alone.

In the course of the same experiment a 3 R culture, obtained by growing a typical Type III S pneumococcus in Type III serum, was injected into a series of eleven mice, together with aliquot portions of the same Type I S vaccine. Seven of the eleven animals succumbed and typical Type I S organisms were recovered from the heart's blood in each instance. All the colonies obtained from this series of animals were of the typical Smooth variety, and no "intermediate" forms were observed. All the cultures possessed maximal virulence for white mice.

Since the heat-killed suspension used in both series was the same the conclusion must be drawn that the atypical intermediate colonies recovered from the first series of mice developed from the 2 R culture and not from the vaccine. This observation offers still further proof of the absence of viable forms in the heat-killed suspensions.

In certain other experiments the injection of an R culture together with a heterologous S vaccine resulted in the recovery of a mixture of S forms from the animals. Such mixtures were composed exclusively of S organisms of the same type as the vaccine and the type from which the R forms had been originally derived. In all such cases S organisms were not obtained from any of the control mice. It must therefore be concluded that, in these instances, conditions were equally suitable for reversion of the R forms to the S forms of the original type, or to the type of the vaccine.

When a plate is composed of a mixture of colonies of various S types the Type III colonies are usually readily identified by their large size and clear, watery appearance. It is also possible, as a rule, to identify colonies composed of Type IS organisms. When examined against a dark background through a plate culture microscope Type I colonies are usually denser, more opaque and whiter than Type II colonies. Colonies composed of Type II and Group IV organisms, on the other

hand, are paler, more transparent and "watery." They also appear to undergo autolysis more readily than Type I colonies and frequently present a 'ring' or 'life-saver' appearance.

Experiments were next undertaken to determine whether one and the same R culture could be successively transformed into the S form of each of the specific types of pneumococcus.

A 2 R culture was obtained by growing a typical Type II pneumococcus in its homologous immune serum. After four transfers the culture was plated. A single R colony was selected and subcultured in blood broth. A single-cell strain was then obtained from this culture by the method of Avery and Leland (4). Four mice were injected with 0.25 cc. of the single-cell culture together with the bacteria from 100 cc. of a Type III S culture heated for 30' at 60°C. Three of the animals succumbed, and typical Type III S pneumococci, possessing all the characteristics of that type, were obtained from the heart's blood of each. One of these III S cultures was then converted into the R form by growth in Type III serum. After five transfers the culture was plated. A single R colony was selected and subcultured in blood broth. The resultant growth was plated and again a single colony was selected and subcultured. This process was repeated four times and the final culture was injected into four mice together with a Type I S vaccine heated for 30' at 60°C. All four mice died and typical Type I S organisms were recovered from the heart's blood in each instance. One of these typical Type I S cultures was again converted to the R form in the same manner as previously described. The resulting R culture was injected into four mice together with a vaccine prepared from a Group IV S culture. Two of the mice died yielding S organisms in the heart's blood. Cultures from the heart's blood of these two animals did not agglutinate specifically in Types I, II or III sera but were highly virulent for mice. Specific anti-serum was not available to test the agglutination of the Group IV S strains, but in view of the preceding results it was highly probable that the cultures were of the same variety as the Group IV vaccine.

During the various stages of this experiment whenever an R culture was obtained it was grown in media containing 10 per cent anti-R serum. In all cases, after a variable number of transfers, the R form was converted to the S form of that type from which it had last been derived. This observation lends support to the contention that in no instance was a mixture of R forms, derived from S forms of more than one type, present in the culture.

In summary, a typical Type II S pneumococcus was successively transformed, through the intermediate stage of the R form, into a Type III S pneumococcus, a Type I S pneumococcus, and a Group

TABLE II

*The Effect of the Temperature at Which a Type I S Vaccine is Heated upon Its Efficacy in Inducing Transformation of Type*

Type and amount of vaccine	Temp. at which vaccine was heated for 15'	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
					R and S forms	N mice
Bacteria obtained from 100 cc. Type I S culture	C.					
	60°	Nil	4	All survived; sacrificed 8-10 days	All cultures negative	4
ditto	60°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	65°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	65°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	70°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	70°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	75°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	75°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	80°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	80°	0.25 cc. 2R	5	All died 1-2 days	Type I S	5
ditto	100°	Nil	2	Both survived; sacrificed 8-10 days	All cultures negative	2
ditto	100°	0.25 cc. 2R	5	Three died 1-2 days	R only	3
				Two survived; sacrificed 8-10 days	All cultures negative	2

IV S pneumococcus. At any stage of the cycle the R form could be converted to the S form of that type from which it had last been derived by growth in anti-R serum.

*The Effect of the Temperature at Which an S Vaccine Is Heated upon Its Efficacy in Causing an R Culture, Derived from a Heterologous S Type<sup>2</sup> to Revert to the Type of the Vaccine*

Griffith reported (2) that S vaccines, when heated at temperatures higher than 70°C., were rarely effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine. To verify this finding the following experiment was devised.

The bacteria obtained by centrifuging 4600 cc. of a Type I S culture were suspended in 23 cc. of plain broth. This suspension was divided into four equal portions of 3.5 cc. each, and two portions of 4.5 cc. each. (The larger portions were heated at the lower temperatures and a greater quantity of suspension was required for additional controls.) Each of these six portions of the same vaccine was heated for fifteen minutes at varying temperatures between 60°C. and 100°C., as outlined in the accompanying table. After heating, a volume of 2 cc. from each of the larger samples, and 1 cc. from each of the smaller samples was reserved for injection into control mice. To the remaining amounts of each portion of vaccine 1.25 cc. of a blood broth culture of a 2 R pneumococcus was then added. The quantities were so arranged that all mice, including the controls, received the heat-killed bacteria from 100 cc. of culture. In addition, each test animal received 0.25 cc. of living 2 R culture.

The results of the experiment appear in Table II.

*Abstract of Protocol.*—Type I S vaccine, heated for a period of 15' at temperatures between 60°C. and 80°C., when injected subcutaneously in white mice together with a living 2 R culture, apparently possesses the ability to cause the 2 R culture to revert to a Type I S pneumococcus. Type I S vaccine heated for 15' at 100°C. does not possess this property.

Attention should be called to certain features in this experiment. In the first place an unusually large number of transformations was obtained. Other experiments were not so uniformly successful. In another experiment a Type I S vaccine, heated for 15' at 70°C. caused a 2 R culture to revert to the type of the vaccine but did not do so when heated for 15' at 80°C. Again, in the experiment recorded above, the Type I S vaccine heated at 100°C. apparently had no effect on the 2 R culture. It did not even cause the R culture to revert to its original S type. In other experiments, however, a Type I S vaccine, heated for 15' at temperatures of 90° and 100°C., caused both 2 R and 3 R cultures to revert to their original S types. The results of such an experiment are reported in Table III.

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<sup>2</sup> More correctly this should read "derived from the S form of a heterologous type." For the sake of brevity in this and succeeding instances the above expression has been adopted.

*Abstract of Protocol.*—In the experiment detailed in Table III Type I S vaccine, heated for 15' at 70°C., was effective in causing a 2 R culture to revert to a Type I S pneumococcus. When heated for the same period at a higher temperature than 70°C. Type I S vaccine was not effective in causing a 2 R culture to revert to the type of the vaccine. However, in several mice which received the Type I S vaccine heated at 80°, 90° and 100°C. the 2 R forms reverted to their original S type.

TABLE III

*The Effect of Temperature at Which a Type I S Vaccine Is Heated upon Its Efficacy in Inducing Transformation of Type. (Second Experiment)*

Type and amount of vaccine	Temp. at which vaccine was heated for 15'	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
					R and S forms	No. mice
Bacteria obtained from 80 cc. Type I S culture.	C.					
ditto	60°	Nil	4	All survived; sacrificed 7 days	All cultures negative	4
ditto	60°	0.25 cc. 2 R	4	All died 1-2 days	Type I S R only	3
ditto	70°	Nil	2	Both survived; sacrificed 7 days	All cultures negative	2
ditto	70°	0.25 cc. 2 R	4	All died 1-2 days	Type I S R only	3
ditto	80°	Nil	2	Both survived; sacrificed 7 days	All cultures negative	2
ditto	80°	0.25 cc. 2 R	4	3 died 1-2 days; 1 survived	Type II S R only	2
ditto	90°	Nil	2	Both survived; sacrificed 7 days	All cultures negative	2
ditto	90°	0.25 cc. 2 R	4	All died 1-2 days	Type II S R only	2
ditto	100°	Nil	2	Both survived; sacrificed 7 days	All cultures negative	2
ditto	100°	0.25 cc. 2 R	4	3 died 1-2 days; 1 survived	Type II S R only	2

Similar results were obtained when a 3 R culture was injected into mice together with a Type I S vaccine heated at various temperatures. From the majority of the animals which received the Type I S vaccine heated at temperatures up to 80°C. S organisms of the same type as that of the vaccine were obtained. From the animals which received the Type I S vaccine heated at higher temperatures than

80°C. no Type I S organisms were recovered. Several of these mice, however, did yield typical Type III S pneumococci.

These findings may be summarized as follows:--Type I S vaccine, heated for 15' at various temperatures between 60° and 80°C. is effective in causing an R culture derived from a heterologous S type

TABLE IV

*The Effect of the Temperature at Which Types II S and III S Vaccines Are Heated upon Their Efficacy in Inducing Transformation of Type*

Type and amount of vaccine	Temp. at which vaccine was heated for 15'	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
					R and S forms	No. mice
Bacteria obtained from 90 cc. Type II S culture.	C.					
	60°	0.25 cc. 1 R	6	3 died 3 survived	Type II S	3
ditto	60°	0.25 cc. 3 R	8	All died	Type II S	8
ditto	100°	0.25 cc. 1 R	6	All survived		
ditto	100°	0.25 cc. 3 R	6	5 died 1 survived	Type III S	5
Bacteria obtained from 90 cc. Type III S culture.	60°	0.25 cc. 1 R	6	4 died 2 survived	Type III S	4
ditto	60°	0.25 cc. 2 R	6	4 died 2 survived	Type III S	4
ditto	100°	0.25 cc. 1 R	6	All survived		
ditto	100°	0.25 cc. 2 R	6	3 died 3 survived	Type II S	3

to revert to the type of the vaccine. When heated at temperatures higher than 80°C. Type I S vaccine does not cause an R culture derived from a heterologous S type to revert to the type of the vaccine; but frequently causes the R culture to revert to its original S type.

Experiments were then undertaken to determine whether vaccines prepared from S organisms other than Type I were subject to the same thermal differentiation as a Type I S vaccine.

(In the previous paper it was shown that vaccines prepared from cultures of Types II S and III S pneumococcus, whether heated at 60°C. or 100°C., were equally effective in causing R organisms, derived from the same S type as that of the vaccine, to revert to their original S type. It was also shown that vaccines prepared by heating cultures of Type I S pneumococcus at 60°C. were effective in causing a 1 R culture to revert to its original S type. However, when a Type I S vaccine was heated at 100°C. this property was destroyed and reversion failed to occur.)

Vaccines were prepared by heating a culture of Type II S pneumococcus at temperatures of 60°C. and 100°C. Equal portions of each lot of vaccine were injected into two series of mice together with 1 R and 3 R cultures, respectively. Similarly, vaccines were prepared by heating a culture of Type III S Pneumococcus at 60°C. and at 100°C. Two series of mice were injected with these vaccines together with 1 R and 2 R cultures, respectively.

The results of these experiments appear in Table IV.

From the foregoing results and from those obtained in previous experiments the following conclusions may be drawn:—

(1) Vaccines prepared by heating cultures of each of the three S types of pneumococcus at 60°C. are effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(2) Vaccines prepared by heating cultures of each of the three S types at 100°C. are not effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(3) Vaccines prepared by heating cultures of each of the three S types at 100°C. are frequently effective in causing 2 R and 3 R cultures to revert to the same specific type from which they were originally derived.

(4) Vaccines prepared by heating cultures of any S type, including Type I, at 100°C. are not effective in causing a 1 R culture to revert to its original S type.

*The Effect of the Duration of Heating upon the Efficacy of a Type I S Vaccine in Causing an R Culture, Derived from a Heterologous S Type, to Revert to the Type of the Vaccine*

Griffith reported that vaccines heated for long periods, even at temperatures as low as 60°C., were less effective than vaccines heated for short periods in causing R forms, derived from heterologous S types, to revert to the type of the vaccine. To test the effect of heating an S vaccine for varying periods the following experiment was arranged.



The bacteria obtained by centrifuging 4000 cc. of a Type I S culture were taken up in 20 cc. of plain broth and heated for 15' at 60°C. A quantity of 8 cc. of suspension was withdrawn and used in the first part of the experiment. The remaining 12 cc. of suspension were heated for a further period of 15' at the same temperature. Three cc. were then withdrawn and a like amount at half-hour intervals thereafter on three occasions. Five cc. of the first 8 cc. sample withdrawn were used for control purposes, a volume of 0.5 cc. being injected subcutaneously into each of ten mice. To each of the five 3 cc. samples 1.5 cc. of a living 2 R

TABLE V

*The Effect of the Duration of Heating upon the Efficacy of a Type I S Vaccine in Inducing Transformation of Type*

Type and amount of vaccine	Length of time during which vaccine was heated at 60°C.	Amount of living R culture	Number of mice	Result	Pneumococci recovered by culture	
					R and S forms	No. mice
Bacteria obtained from 100 cc. Type I S culture.	15'	Nil	10	All survived; sacrificed at intervals up to 17 days	All cultures negative	10
ditto	15'	0.25 cc. 2 R	6	5 died 1 survived	Type I S	5
ditto	30'	0.25 cc. 2 R	6	4 died 2 survived	Type I S	4
ditto	1 hour	0.25 cc. 2 R	6	5 died 1 survived	Type I S	5
ditto	1½ hours	0.25 cc. 2 R	6	4 died 2 survived	Type I S	4
ditto	2 hours	0.25 cc. 2 R	6	5 died 1 survived	Type I S	5

culture was added. Six test mice were inoculated in each part of the experiment with the heat-killed bacteria from 100 cc. of Type I S culture, together with 0.25 cc. of living 2 R culture.

The details of the experiment are given in Table V.

*Abstract of Protocol.*—Type I S vaccine, heated for as long a period as two hours at 60°C., when injected subcutaneously in white mice together with a living 2 R culture, apparently possesses the ability to cause the 2 R forms to revert to Type I S organisms. A Type I S vaccine heated for 2 hours at 60°C. is just as effective in producing reversion to the type of the vaccine as that heated for 15' at 60°C.

Mention should again be made of the fact that this also was an unusually successful lot of vaccine. In other experiments a smaller number of positive results was obtained and there appeared to be a slight falling off in the effectiveness of the vaccine when heated for prolonged periods at 60°C.

*The Amount of Heat Killed S Organisms Necessary to Cause R Forms Derived from a Heterologous S Type, to Revert to the Type of the Vaccine*

The minimal amount of vaccine capable of inducing transformation of type was then ascertained. An experiment was devised in which

TABLE VI

*The Amount of Heat-Killed Suspension Necessary to Induce Transformation of Type*

Amount of culture from which Type I S vaccine was prepared	Amount of living R culture	Number of mice	Result	Pneumococci recovered on culture	
				R and S forms	No. mice
100 cc.	0.25 cc. 2 R	4	4 died	Type I S	4
50 cc.	0.25 cc. 2 R	4	3 died 1 survived	Type I S	3
25 cc.	0.25 cc. 2 R	4	2 died 2 survived	Type I S	2
10 cc.	0.25 cc. 2 R	4	All survived	—	
100 cc.	Nil	4	All survived; sacrificed 7 days.	All cultures negative.	4

four series of mice received a living 2 R culture together with varying amounts of Type I S vaccine. In addition to the living R culture the first series of animals received the heat-killed bacteria from 100 cc. of S culture; the second from 50 cc.; the third from 25 cc. and the fourth from 10 cc. Four control mice received only the heat-killed bacteria from 100 cc. of S culture.

The results of this experiment appear in Table VI.

*Abstract of Protocol.*—From the heart's blood of all 4 mice which received the living 2 R culture together with the heat-killed bacteria from 100 cc. of Type I S culture, S organisms of the same type as the vaccine were obtained. Similarly

Type I S organisms were recovered from 3 out of 4 mice which received the heat-killed bacteria from 50 cc. and from 2 out of 4 which received the bacteria from 25 cc. From those which received the bacteria from 10 cc. no Type I S organisms were obtained. The control mice which were injected with the heat-killed bacteria from 100 cc. of Type I culture all survived. They were sacrificed at the end of 7 days and cultures from the blood and viscera were negative.

In many other experiments it has constantly been found that large amounts of vaccine are necessary to effect transformation of type by this procedure.

*The Effect of Autolysis on the Efficacy of an S Vaccine in Causing R Forms, Derived from Heterologous S Types, to Revert to the Type of the Vaccine*

Early in the work it was found that many lots of vaccine were relatively ineffective in causing R forms to revert to the Type of the vaccine. In searching for an explanation of these failures it was noticed that many mice in such unsuccessful experiments developed purpura to a marked degree. Julianelle and Reimann (5) have shown that the purpura-producing fraction of pneumococcus is released only during autolysis and is not present in heat-killed cultures in which autolysis has not taken place. This fact suggested a possible explanation for the ineffectiveness of certain lots of vaccine. It seemed possible that the cultures might have undergone partial autolysis before being heat-killed and, as a consequence, the vaccines made from such cultures were no longer effective in producing reversion. To test this hypothesis the following experiment was done:—

Cultures of Type I S and Type II S organisms were centrifuged and the deposit divided into two equal portions. One-half of the deposit from each culture was immediately heated at 60°C. for 15': the other half was allowed to autolyze at 37°C. for 48 hours. At the end of this period the autolysate was subjected to a temperature of 60°C. for 15'. The two preparations were injected into a series of mice together with cultures of heterologous R organisms as detailed in Table VII.

*Abstract of Protocol.*—Autolysates of S cultures, when injected subcutaneously in white mice together with living R cultures, are not effective in causing R forms to revert, either to their original S type, or to the S type from which the autolysate was prepared. (Heat-killed suspensions of S organisms, however, kept in the ice-box for periods up to three weeks, have been found to be effective in causing R forms derived from heterologous S types, to revert to the type of the vaccine.)

This experiment offers evidence that it is not the specific soluble substance as such that is responsible for transformation of type in these procedures. The specific soluble substance of pneumococcus is not altered during autolysis and is present both in the autolysate and in the heat-killed suspension (6).

TABLE VII

*The Effect of Autolysis on the Efficacy of an S Vaccine in Inducing Transformation of Type*

Type and amount of vaccine	Type and amount of autolysate	Amount of living R culture	Number of mice	Result	Pneumococci recovered on culture	
					R and S forms	No. mice
Bacteria obtained from 80 cc. Type I S culture heated for 15' at 60°C.	Nil	0.25 cc. 2 R	4	4 died	Type I S	4
ditto	Nil	0.25 cc. 3 R	4	3 died 1 survived	Type I S	3
Nil	Bacteria obtained from 80 cc. Type I S culture allowed to autolyze for 48 hrs. at 37°C.	0.25 cc. 2 R	4	All survived; sacrificed 7 days.	All cultures negative	4
Nil	ditto	0.25 cc. 3 R	4	All survived; sacrificed 7 days.	All cultures negative	4

*The Effect of Injecting S Cultures Killed by Other Agents than Heat Together with R Cultures Derived from Heterologous S Types*

In the experiments reported up to this point the S cultures were invariably killed by heat.

In order to determine whether or not S organisms killed by other agents than heat possessed similar properties, cultures of S pneumococci were killed with the following substances:—formalin, iodine,

chloroform, tricoresol, alcohol and acetone. None of the reagents selected destroy the specific soluble substance of pneumococcus (7). The smallest possible concentrations were employed to eliminate the toxic effect of the reagents themselves.

Six series of five mice each were injected with a living 2 R culture together with the bacteria from 100 cc. of Type I S culture which had been killed by adding minimal amounts of each of the above mentioned bactericidal substances. Reversion to the type of the vaccine did not occur in a single instance. At the same time five animals were injected with the bacteria from 100 cc. of the same Type I S culture, heated for 15' at 60°C. together with the same living 2 R culture. Type I S organisms were recovered from the heart's blood in each instance. Four control mice which received only the heat-killed vaccine survived.

The failure to induce reversion by the use of suspensions of S organisms killed by other agents than heat suggests one of two possibilities;—either that portion of the vaccine responsible for reversion may have been destroyed by the bactericidal substances; or, the toxic effect of the reagents may have created unfavorable conditions in the tissues of the animals.

*Results Obtained by the Intraperitoneal Injection of Small Amounts of Living R Forms Together with the Heat-Killed Bacteria from Large Amounts of Heterologous S Cultures*

In all the preceding experiments living R forms and S vaccines were injected *subcutaneously* in white mice. Experiments were undertaken to determine whether similar transformations could be effected by the *intraperitoneal* injection of living R forms together with vaccines of S cultures. It was hoped that it would be possible during this procedure to follow the transformation process by withdrawing and examining portions of the peritoneal contents from time to time. The results were not uniformly satisfactory. In one experiment eight mice were injected intraperitoneally with a living 2 R culture together with a Type III S vaccine. Four of the animals died and Type III S organisms were recovered from the heart's blood. However, these organisms were only slightly agglutinable in Type III serum and the colonies were not of the large, typical Type III S variety. Typical Type III organisms were obtained by passing these cultures through second

mice. The first cultures were apparently composed of incompletely developed Type III S forms.

In another experiment varying amounts of a living 2 R culture were injected intraperitoneally together with large quantities of a Type I S vaccine. In no case did the R form revert to the type of the vaccine. It was therefore concluded that, while reversion to the type of the vaccine could be effected intraperitoneally, the subcutaneous route was the method of choice.

*Attempts to Determine the Minimal Time Required to Effect Reversion of R Forms into Organisms of Heterologous S Type*

In different experiments there was a great variation in the interval required to effect reversion. The shortest time in which an animal succumbed, yielding S organisms of the same type as that of the vaccine, was eighteen hours; the longest nine days. In some experiments virulent S organisms were recovered at the site of injection from apparently healthy animals which were sacrificed at the end of seven days. The finding of virulent bacteria in local abscesses in otherwise healthy animals after such an interval suggested that the mice had acquired considerable general immunity before the R forms had had the opportunity to develop into the S form. In the most successful experiments the usual time at which the animals succumbed was from one and one-half to two days after injection. However, if, in this period, S forms had developed in sufficient numbers to overwhelm the animal the actual time necessary for the reversion process to occur at the site of injection was probably much shorter. An attempt was made to determine the minimal time required in the following way.

A series of mice was injected subcutaneously with an S vaccine. Thereafter, at intervals of two, four, eight, twelve, and twenty-four hours living R forms were introduced into the same animals at the same location. Reversion to the type of the vaccine occurred in a moderate number of animals which received the living R forms as late as eight hours after the injection of the vaccine. Reversion did not occur in any animal which received the living culture after an interval longer than eight hours. However, only a small number of animals was employed in this experiment and for this reason the results cannot be considered conclusive. The procedure of introducing the living R forms simultaneously with the vaccines uniformly gave a higher proportion of positive results.

*Attempts to Effect Reversion by the Injection of Living R Forms and S Vaccines in Different Locations in the Same Animals*

Various ways in which S vaccines might act in causing reversion of R forms in the animal body were considered. One of the possibilities was that the general conditions created in the animal by the injection of such large amounts of vaccine might be suitable for the development of organisms of the S variety. If the reversion process depended upon the existence of such general conditions it might be possible to produce a comparable state by the injection of S vaccines and living R forms into different locations in the same animals. Six mice were injected with a Type I S vaccine in one inguinal region. Simultaneously a living 2 R culture was injected into the opposite inguinal region. One of the six mice died in two days and organisms of the same type as the vaccine were recovered from the heart's blood. The other five mice survived. However, such experiments are not conclusive for it is always possible that no matter where the living R forms are injected some organisms may find their way to the site of the vaccine. Because of this difficulty no further experiments were attempted along these lines.

*Attempts to Effect Reversion by the Injection of Living R Forms of Pneumococcus Together with S Vaccines of Friedländer's Bacillus*

It has been demonstrated in this laboratory that Type II pneumococcus and Type B Friedländer's bacillus elaborate specific soluble substances which are chemically and immunologically similar, although not identical (8). Experiments were therefore undertaken to determine whether it was possible to convert R pneumococci, derived from other S types than Type II, into Type II S organisms, by the use of a Friedländer Type B vaccine. R forms of pneumococci, derived from both Type I S and Type III S organisms, were injected into a series of mice together with a Type B Friedländer vaccine. The experiments were accompanied by certain difficulties, for it was found that the primary toxicity of S Friedländer vaccine was considerable.

In the first experiment a series of four mice was injected with the bacteria from 80 cc. of Type B Friedländer culture along with a living culture of 3 R pneumococcus. A similar number of animals was injected with the same Friedländer

vaccine and a culture of 1 R pneumococci. All the animals died after an interval of less than twenty-four hours but only R forms of pneumococci were recovered from the heart's blood.

The converse of the above experiment was then done.

R forms of Friedländer bacilli were injected into mice along with the heat-killed bacteria from a Type II S pneumococcus culture. R forms derived from each of the three specific types A, B, C, and from a strain of the heterogeneous group X were employed. Four mice were injected in each experiment. Two animals died after a period of twelve hours; the remainder survived and were sacrificed after a period of six days. Cultures made from the site of injection and from the heart's blood did not yield S Friedländer bacilli in a single instance. R forms were found at the site of injection in a large proportion of cases. Cultures of the recovered R forms were passed through a second series of mice and the virulence of the organisms was found to remain unchanged. Unfortunately many of the mice in this experiment developed ulcers at the place of injection. It is possible that this fact may have had some effect in determining the results obtained. It is also possible that the particular R strains of Friedländer's bacillus which were selected were not suitable for reversion.

#### *Attempts to Convert R Forms of Pneumococci into Organisms of the Heterologous S Type by in Vitro Methods*

The *in vitro* methods which were employed in attempts to convert R forms into organisms of the homologous S type, by the use of vaccines, have been described in a preceding paper (1). All the procedures adopted gave negative results. Similar attempts were made to convert R forms into S organisms of heterologous types. All such experiments were unsuccessful. It must therefore be concluded that, either the *in vitro* conditions, as provided, were inadequate, or that living tissues are essential for the reversion process. An attempt to partially reproduce *in vivo* conditions in the test tube was made in the following way.

Large quantities of a Type I S vaccine were injected intraperitoneally into five mice. The animals were sacrificed at intervals of 2, 4, 8, 12 and 24 hours and the peritoneal contents washed out with plain broth. The washings were transferred to test tubes and seeded with a 2 R culture. Plates made from the resulting growth, however, yielded only R colonies and the cultures remained avirulent for white mice.



The negative results of this experiment suggest that living tissues may play a part in the reversion process. However, it should be pointed out that in previous experiments the intraperitoneal route was not found as suitable as the subcutaneous route. The possibility remains that if the conditions obtaining in the subcutaneous tissues of the mouse could be reproduced in the test tube transformation of type might be effected *in vitro*.

*Attempts to Convert S Pneumococci of One Specific Type Directly into S Organisms of Another Specific Type*

In all the experiments described in which pneumococci have been converted from one specific S type into other specific S types the transformation has been effected through the intermediate stage of the R form. Inasmuch as the phenomenon of transformation of type had never been observed in type-specific S cultures under artificial cultivation it seemed most unlikely that S organisms could be transformed directly from one specific type to another specific type. An attempt to effect such a direct transformation of type was made in the following manner. Mice were injected subcutaneously with the smallest possible dilutions of living S cultures together with the heat-killed bacteria from large amounts of heterologous S cultures. For example a Type II S culture, in dilutions of  $10^{-7}$ ,  $10^{-8}$ , and  $10^{-9}$  cc. was injected into a series of mice together with a Type I S vaccine. In all cases the animals succumbed and only S organisms of the same type as those introduced in the living cultures were recovered from the heart's blood. Direct transformation from one type to another did not occur in a single instance. These experiments also prove that large quantities of vaccine have no inhibitory effect on any viable forms introduced with the vaccine. On the contrary the animals that received both the living culture and the vaccine succumbed in a much shorter period of time than those which received only the dilutions of living culture.

#### DISCUSSION

The transformation of pneumococci from one specific type into other specific types is a phenomenon of wide bacteriological and epidemiological significance. It has not been conclusively demonstrated

that transformation of type actually occurs under natural conditions. Griffith (2) has presented certain evidence which indicates the possibility of such an occurrence during disease processes, but further work is required to establish definitely the validity of these observations. In any case the demonstration that transformation of type may be effected experimentally shows that the various types of pneumococcus are closely related biologically. Indeed it is possible to think that these various types may represent attempts on the part of the organism to adapt itself to varying environmental conditions.

It is important to note that it was found impossible to transform S organisms directly from one specific type into other specific types. Change of type was invariably brought about through the intermediate stage of the R form. The R form of the organism is most readily produced by growing S organisms in their homologous immune serum. It may also be produced by subjecting S pneumococci to unfavorable environmental conditions,—such as, growth in poor media, growth at temperatures between 40° and 42°C., and growth in media containing small traces of bile. The R form, therefore, probably results from attempts of S bacteria to adapt themselves to unfavorable environmental conditions. Once reduced to the R state the organisms potentially have the capacity to develop the S structure of any of the various specific S types. They most readily assume the characteristics of that S type from which they were last derived; but under the influence of certain conditions they may also develop the S structure of other specific types.

What conditions determine the development of S characteristics? The change may be induced experimentally by subcutaneously injecting, in white mice, large amounts of an S vaccine together with living R forms. The type of S structure which the R forms assume under these conditions is apparently dictated by two circumstances; (1) The degree of "degradation" to which the R forms have been subjected; (2) The degree of heat to which the S vaccine has been exposed. If the R forms have been reduced to a definite state in the "degradation" process they assume the characteristics of the same S type as the vaccine. If the R forms have been only partially "degraded" they assume the characteristics of that S type from which they were originally derived. Similarly, if the vaccine is heated at a tempera-

ture between 60° and 80°C. the R forms revert to the type of the vaccine: if the vaccine is heated at a temperature higher than 80°C. the R forms revert to the S type from which they were originally derived.

What are the causes responsible for transformation of type as induced by this procedure? In the previous paper various possibilities were considered to explain the way in which S vaccines might act in causing R forms, derived from the same S type as the vaccine, to revert to their original S type. It was pointed out that the precise factor responsible for reversion, as brought about by this procedure, was not understood. If the causes determining reversion of R forms to their original S type are not understood it is even more difficult to interpret the conditions under which R forms assume the characteristics of S organisms of the same type as the vaccine. That property of the vaccine responsible for reversion does not exactly correspond with any known substance or property of S organisms. It cannot be the S substance itself, for it has been shown that the carbohydrate fraction of pneumococcus is not altered by heating at 100°C. (9), and its specificity is not destroyed during autolysis. Moreover, the efficacy of an S vaccine in inducing transformation of type does not parallel the antigenic properties of the vaccine. It has been shown that vaccines of S pneumococci are equally good antigens whether heated at 60°C. or at 100°C.

It is possible that S vaccines may exert their effect in one of two ways:—(1) Directly on the R forms themselves; (2) On the tissues of the animals in which they are injected.

The failure of all *in vitro* attempts to secure transformation of type suggests that, if the vaccine exerts its influence directly on the living R forms, it does so only under very precise conditions. If it were possible to reproduce in the test tube the conditions obtaining in the subcutaneous tissues, of the mouse, transformation of type might be effected *in vitro*. However, it would be difficult to duplicate experimentally the conditions created by the disintegration and digestion of large amounts of vaccine in the living tissues of an animal.

A second possibility is that the conditions created in the subcutaneous tissues of the mouse offer a suitable environment in which the R forms may build up their S structure. In the previous paper it was pointed out that, under natural conditions, the white mouse "possessed

some capacity to overcome infection by organisms producing minimal amounts of S substance." It was further suggested that "the injection of S vaccines might destroy or inhibit this limited ability of the mouse and under such conditions the R forms might develop into S organisms." May it not be possible that the injection of an S vaccine only inhibits or destroys the capacity of the mouse to overcome infection by that particular S type? Under such circumstances may not the R organism, potentially capable of synthesizing any type of polysaccharide, be able to elaborate that particular S substance most suitable for the survival of the organism in its environment?

In this connection reference is again made to the work of Sia (10). Employing serum-leucocyte mixtures in a specially constructed apparatus, he reported the following observation.

"The presence of a small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixtures so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which ordinarily possess the power to destroy such pneumococci in relatively large numbers." Sia further reported that this effect was highly type-specific for "a Type II substance assisted the growth of only pneumococcus Type II; likewise a Type III substance, the growth of pneumococcus Type III only."

Any such explanation, however, fails to account for the different effects produced by vaccines heated at temperatures above and below 80°C. Further work is therefore required to understand clearly the causes responsible for transformation of type as induced by Griffith's technique.

In the previous paper it was pointed out that R forms of pneumococcus could be found in the flora of the upper respiratory tract of many normal individuals. It was suggested that these forms resulted from attempts of the bacteria to adapt themselves to unfavorable environmental conditions. Although degraded to the R form these organisms still retained the capacity of again developing into virulent, type-specific, S pneumococci. Any such development would appear to be dictated by conditions in the environment. Those environmental conditions would also determine the particular S type which the R organisms may assume. In the absence of more precise data concerning such transformations further speculation is unprofitable. However, the possibilities of alteration in type under natural and

disease conditions cannot be ignored and may attain proportions of much significance in infectious and epidemiological problems.

### CONCLUSIONS

1. Type-specific S pneumococci may be transformed from one specific S type into other specific S types through the intermediate stage of the R form.

2. R forms of pneumococci, derived from any specific S type, may be transformed into S organisms of other specific types by the following procedure:—The subcutaneous injection, in white mice, of small amounts of living R forms together with vaccines of heterologous S cultures.

(i) S vaccines heated for 15' at temperatures between 60° and 80°C., are effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(ii) S vaccines heated for 15' at temperatures between 80° and 100°C., are not effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(iii) S vaccines heated for 15' at temperatures between 80° and 100°C., may cause 2 R and 3 R cultures to revert to their original S type.

(iv) S vaccines of any type, including Type I, heated for 15' at temperatures between 80° and 100°C., are not effective in causing 1 R cultures to revert to their original S type.

(v) S vaccines heated for periods as long as two hours at 60°C. are effective in causing R forms, derived from heterologous types, to revert to the type of the vaccine.

3. A single cell R strain, derived from a Type II S pneumococcus, has been successively transformed into a Type III S, a Type I S and a Group IV S culture.

4. Corresponding with the various degrees of "degradation" of the R form there are varying degrees of "development" of the S form.

5. The nature of the conditions responsible for alteration of type as induced by these procedures has been investigated and the causes responsible for the transformations are discussed.

6. All attempts to produce transformation of type *in vitro* have been unsuccessful.

7. The rôle which the phenomenon of transformation of type may play in problems of infection and epidemiology is indicated.

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## SURVIVAL OF VACCINE VIRUS SEPARATED FROM LIVING HOST CELLS BY COLLODION MEMBRANES

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The fact that vaccine virus is able to multiply in the presence of living host cells is well established (1-5). None of the reports, however, regarding its increase in lifeless media has received general confirmation. Harde (6), using tissue cultures for the growth of vaccine virus, showed that the infectious agent was principally associated not with the plasma clot but with the cells. Parker and Nye (2) using an improved technique made observations similar to those of Harde. Although it is generally recognized that living host cells are essential for the *in vitro* multiplication of vaccine virus and are intimately associated with the active agent, no one definitely knows what function they serve in the cultures.

For many years collodion membranes, in one form or another, have been used in bacteriology. Attempts have been made to study the relation of viruses to host cells by implanting collodion sacs containing the active agents in the peritoneal cavity of experimental animals. Williams and Fournoy (7) investigated vaccine virus in this manner and found that it survived only for a short time. Olitsky and McCartney (8) implanted the virus of typhus fever in the peritoneal cavity of guinea pigs and reported that it survived for 31 days. The objection to such experiments is that the collodion sacs with their contents acted as foreign bodies, so that, instead of the viruses being brought into close relation with normal host cells, they were brought into an indirect contact with fibrous capsule by which the bags became surrounded. This difficulty might have been avoided through the use of tissue cultures, were it not for the fact that in the past most of the techniques of such culture required the use of solid media in the form of plasma clots.



Recently Maitland and Maitland (9) reported that vaccine virus is capable of multiplication in a fluid medium consisting of a mixture of one third serum and two thirds Tyrode's solution in which bits of finely minced kidney tissue are suspended. These observations have been confirmed (10, 11). Such a fluid medium, in which living cells persist for at least 5 days (12), makes possible the use of semi-permeable membranes in investigations concerning the functions served by living host cells in the survival and multiplication of viruses.\* In the present paper are reported observations on the survival of vaccine virus separated from living cells by collodion membranes.

### *Methods and Materials*

*Vaccine Virus.*—The neurovaccine virus of Levaditi was used. The testicles of rabbits, inoculated with a 1-10 dilution of the virus, were removed on the fourth day, emulsified in Locke's solution with sand, and centrifuged at high speed for 20 minutes. The supernatant fluid was used as an inoculum. The final dilution of the inoculum was identical in all of the preparations at the beginning of each experiment.

*Tests for Vaccine Virus.*—At the completion of each experiment, tests for the presence of active vaccine virus were made by emulsifying the different mixtures in a mortar without sand and injecting 0.25 cc. of each into the shaved skin of a rabbit.

*Dialyzing Apparatus.*—The dialyzing apparatus consisted of an outer tube flared slightly at the top to fit a shoulder on an inner tube, the two being ground to form a perfect joint (Text-fig. 1). The collodion sac was attached to the lower part of the inner tube by means of a rubber band. When 1 cc. of fluid was placed inside the collodion bag, and from 4 to 4.5 cc. in the outer tube, the fluid level was the same on the two sides of the membrane.

*Preparation of Collodion Sacs.*—The method of preparing the collodion bags is a slight modification of the one used by Northrop and Kunitz (13). Pyrex test tubes, with inside dimensions of 1.5 x 11 cm. were thoroughly cleansed and dried, after which 1.5 cc. of Merck's contractile collodion was placed in each tube. The tubes were then stoppered and allowed to stand until all bubbles had disappeared from the fluid, when the stoppers were removed and the tubes were placed horizontally in holders revolving at the rate of 20 revolutions per minute. After rotating for 5 minutes the tubes were removed from the machine and kept inverted for 2½ to 3 minutes. Then they were filled with water and the sacs were removed by permitting the fluid to run between the sacs and the walls of the tubes.

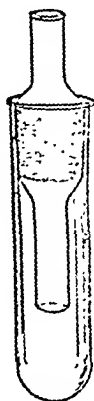
*Tests of Collodion Sacs.*—Each bag was cut to a length of about 6 cm. and the tip

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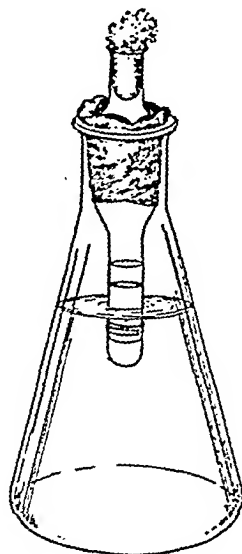
\* Semipermeable membranes have been used by Wollman, Bronfenbrenner, and Muckenfuss in investigations concerning the nature of bacteriophages (see J. Bronfenbrenner and R. Muckenfuss, *Jour. Exp. Med.*, 1927, 45, 887).

of the inner tube of the dialyzing apparatus (Text-fig. 1) was slipped inside the sac until the distance from the end of the bag to the tip of the glass tube was 2.5 cm. The bag was then secured in place by a rubber band, the tube was completely filled with water, and a one-holed rubber stopper to facilitate connection with the testing apparatus described below was inserted in the top of the tube.

The testing apparatus consisted of a narrow glass tube about 1 meter in length at the lower end of which was a 2-way stopcock (Text-fig. 3). In one direction



TEXT-FIG. 1



TEXT-FIG. 2

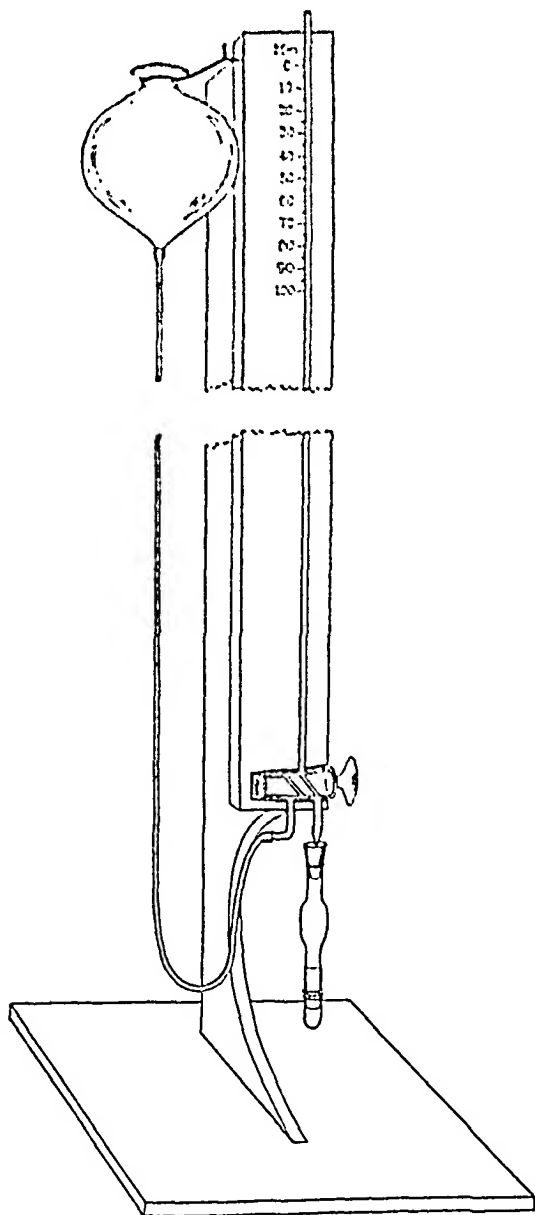
TEXT-FIG. 1. The inner and outer glass tubes of dialyzing apparatus. The collodion membrane fits over the lower part of inner tube and is held in place by an elastic band (see Text-fig. 2).

TEXT-FIG. 2. Manner in which inner tube with its collodion membrane is prepared for sterilization in the autoclave.

this led to a long rubber tube and a levelling bulb, which was filled with water. In the other direction it led to a straight glass tip which was drawn out to fit into the rubber stopper placed in the top of the inner tube of the dialyzing apparatus described above. The distance from the top of the collodion bag to the point on the support of the vertical glass tube, from which measurements were made, was 1 meter.

The apparatus was designed for the determination of the amount of water that would pass through the membrane in a given time under a pressure of 1 meter of water. The water level in the tube was adjusted to 1 meter, and the stopcock was

turned so that pressure was delivered inside the collodion sac. The bag was then examined for leaks and if none were present the amount of water passing through



TEXT-FIG. 3. Apparatus for testing the permeability of the collodion sacs.

in 3 minutes was read on the mm. scale beside the vertical tube. With the tubing used in our work a length of 9 cm. contained 1 cc. of water. Consequently, the

volume of water passing through the membrane was readily calculated. The membranes used permitted from 0.52 cc. to 0.78 cc. to pass through in 3 minutes.

*Sterilization of the Collodion Sacs.*—Upon completion of the tests, the tubes of the dialyzing apparatus were removed, part of the water was withdrawn, and the rubber stopper was replaced by nonabsorbent cotton. The shoulder of each inner tube was then wrapped with cotton strips surrounded by gauze and the tube itself was inserted into a 150 cc. Erlenmeyer flask, the cotton-gauze wrapping serving as a support (Text-fig. 2). The water level in the flask was above the top of the collodion bags but not as high as that inside the tubes. The flasks were then placed in the autoclave and sterilized under 5 pounds pressure for 45 minutes. The outer tubes of the dialyzing apparatus were sterilized by dry heat.

Several sacs were retested after sterilization and their permeability was found to be reduced to about one third of what it was before this treatment. The bags used in the experiments here reported were not again tested after sterilization.

*Maitland Medium.*—For the cultivation of vaccine virus, Maitland used chicken serum, Tyrode's solution, and minced chicken kidney in the following proportions: serum, 6 cc.; Tyrode's solution, 12 cc.; minced kidney, 0.66 cc. In the experiments reported in the present paper the Maitland technique was followed with the exception that rabbit serum and kidney were used.

#### EXPERIMENTAL

For controls on the results of each experiment, data was obtained by testing the survival of vaccine virus alone in test tubes, and its persistence in the presence of living and frozen (last 2 sets of experiments) tissue both in test tubes and in Carrel flasks. For this reason some of tests were repeated more frequently than were others. In all, 24 experiments with collodion bags were performed. These are summarized in Table I which will be referred to from time to time as individual experiments are described.

As a preliminary study it was essential to ascertain whether vaccine virus would pass through the collodion sacs prepared in the manner above described, for, if it did diffuse through their walls, the bags would be unsuitable for the work.

#### *Diffusibility of Vaccine Virus*

A freshly prepared testicular emulsion containing active vaccine virus was placed in the collodion sacs, and Locke's solution was placed in the outer chambers of 4 dialyzing apparatuses, 2 of which were placed in the incubator and 2 in the ice box. After 3 days the fluids on both sides of the sacs were tested for the presence of vaccine virus.

It was found that the virus retained its activity in the ice box and did not diffuse through the membranes. In the incubator the virus lost activity, consequently no data concerning its diffusibility at 37°C. was obtained. From the results of later experiments, however, it became obvious that vaccine virus will not diffuse through the type of membranes used.

The object of the first set of experiments was to determine if the survival of vaccine virus, suspended in a cell-free mixture of serum and Tyrode's solution, is enhanced by the presence of a culture of vaccine virus in Maitland's medium on the opposite side of a collodion membrane.

### *Survival of Vaccine Virus Separated from Living Cells Inoculated with Vaccine Virus*

In attempts to determine the effect that Maitland's medium inoculated with vaccine virus has on the survival of vaccine virus suspended in a cell-free mixture of serum and Tyrode's solution on the opposite side of a semi-permeable membrane, 6 experiments were performed as described below.

2 mixtures were prepared. One consisted of vaccine virus diluted 1-100 by a fluid made of one third rabbit serum and two thirds Tyrode's solution, the other was composed of vaccine virus (final dilution also 1-100) in Maitland's medium.\* The suspension of vaccine virus was then placed on one side of the membranes in the dialyzing apparatuses, and Maitland cultures of vaccine virus on the other. The dialyzing sets were then placed in the incubator at 37°C. for 4 days, at the end of which time the fluids on both sides of the membranes were tested upon rabbits for the presence of active vaccine virus. As controls in each experiment virus (final dilution 1-100) was incubated in test tubes and in Carrel flasks.

The results of the above experiments with those to follow are summarized in Table I and show that vaccine virus alone survived in one third of the test tubes (controls), that it persisted or multiplied in Maitland's medium in all the dialyzing apparatuses, and that it survived in only one half of the dialyzing apparatuses when separated from the Maitland cultures by means of membranes. From these findings it is seen that the virus survived or actually multiplied,

\* It must be remembered that Maitland's medium is prepared by mixing 0.66 cc. of minced normal kidney, 6 cc. of serum, and 12 cc. of Tyrode's solution.

as shown previously (10, 11), when it was in direct contact with living kidney cells, and that the survival of the virus in serum and Tyrode's solution was not influenced to any great extent when the active agent was separated from the Maitland cultures of the virus by means of semi-permeable membranes.

The possibility that the virus might have so severely injured the cells in the Maitland cultures that they were useless for the purposes of the experiment, or that the virus in the Maitland cultures required all of the factors necessary for survival, leaving none to diffuse through the membranes, was next considered. To test this point, uninoculated Maitland medium was placed on one side of the membranes, and vaccine virus, suspended in serum and Tyrode's solution, on the other.

#### *Survival of Virus Separated from Uninoculated Living Tissue*

12 experiments were made to ascertain what effect uninoculated Maitland medium placed in one chamber of the dialyzing apparatus had on the survival of vaccine virus suspended in serum and Tyrode's solution in the other chamber.

Vaccine virus was prepared and diluted with serum and Tyrode's solution as in the previous experiments, and Maitland medium was made in the usual manner. In the dialyzing apparatuses vaccine virus was then placed on one side of the membranes, and uninoculated Maitland medium on the other. After incubation for 4 days at 37°C. material from each side of the membranes was tested for the presence of vaccine virus.

The results of the experiments are summarized in Table I. Out of 12 tests the vaccine virus was found to survive in every case, and no virus was demonstrated in the Maitland medium on the opposite side of the membranes, except in one instance when virus passed through a leak in the bag. When the results of these experiments are examined and compared with those described above, it becomes evident that something from the living cells diffused through the membranes and supported the survival of the virus.

Although the virus survived in every instance, it seems that some of its activity was lost. In efforts to enhance the survival, it was next decided to run experiments similar to the ones just described, with the exception that dead cells (killed by repeated freezing and thawing)

TABLE I  
Summary of Experiments Concerning the Survival of Vaccine Virus at 37°C.

No. of tests	Containers	Contents	Results of tests for vaccine virus	
			Negative	Positive
12	Test tubes	Vaccine virus, serum, Tyrode's solution	9	3 (+ to +++)
12	Test tubes	Vaccine virus in Maitland medium	6	6 (± to +++)
4	Test tubes	Vaccine virus in Maitland medium (frozen)	3	1 (+)
12	Carrel flasks	Vaccine virus in Maitland medium	4	8 (++++ to +++)
2	Carrel flasks	Vaccine virus in Maitland medium (frozen)	0	2 (±)
4	Inner tube	Vaccine virus in Maitland medium	0	4 (++++ to +++)
	Outer tube	Vaccine virus, serum, Tyrode's solution	2	2 (+)
2	Inner tube	Vaccine virus, serum, Tyrode's solution	1	1 (+)
	Outer tube	Vaccine virus in Maitland medium	0	2 (++++ to +++)
6	Inner tube	Uninoculated Maitland medium	5	1 (leak in bag)
	Outer tube	Vaccine virus, serum, Tyrode's solution	0	6 (++++ to +++)
6	Inner tube	Vaccine virus, serum, Tyrode's solution	0	6 (++++ to +++)
	Outer tube	Uninoculated Maitland medium	6	0
3	Inner tube	Vaccine virus in frozen Maitland medium	0	3 (++++ to +++)
	Outer tube	Uninoculated Maitland medium	3	0
3	Inner tube	Uninoculated Maitland medium	3	0
	Outer tube	Vaccine virus in frozen Maitland medium	0	3 (++++ to +++)

Maitland medium consists of minced normal kidney suspended in serum and Tyrode's solution. Inner and outer tubes refer to the tubes of the dialyzing apparatuses. The pluses indicate the amount of vaccine virus present as determined by the severity of the reactions in the skin of rabbits.

were added to the suspensions of vaccine virus in serum and Tyrode's solution.

*Survival of Virus in Contact with Dead Cells When Separated from Living Tissue by a Collodion Membrane*

A fresh emulsion of vaccine virus was prepared in the usual manner, care being taken to remove all cells by centrifugation. Fresh rabbit serum was collected from which all cells were removed. Fresh rabbit kidney was finely minced, half of which was frozen (CO<sub>2</sub> snow) and thawed 10 times.

A mixture of vaccine virus, serum, Tyrode's solution, and frozen kidney tissue was placed on one side of the membranes in the dialyzing apparatuses, and a preparation consisting of normal kidney tissue, serum, and Tyrode's solution on the other. The usual controls were set up. All preparations were incubated at 37°C. for 4 days, after which they were tested for the presence of active vaccine virus by intradermal inoculations (0.25 cc.) in rabbits. The results of the tests are given in detail below and are also summarized in Table I. The pluses indicate the amount of vaccine virus present as determined by the severity of the reaction in the rabbits.

	Set A	Set B
Vaccine virus in test tubes.....	—	—
Vaccine virus, normal kidney, serum, and Tyrode's solution in test tubes.....	—	+±
Vaccine virus, frozen kidney, serum, and Tyrode's solution in test tubes.....	—	—
Vaccine virus, normal kidney, serum, and Tyrode's solution in Carrel flasks.....	++++	++++
Vaccine virus, frozen kidney, serum, and Tyrode's solution in Carrel flasks.....	±	±
Vaccine virus, serum, and Tyrode's solution in inner chamber of dialyzing apparatus.....	++	
Normal kidney, serum, and Tyrode's solution in outer chamber of dialyzing apparatus...	—	
Vaccine virus, serum, and Tyrode's solution in outer chamber.....	+++	
Normal kidney, serum, and Tyrode's solution in inner chamber.....	—	
Vaccine virus, frozen kidney, serum, and Tyrode's solution in inner chamber.....	+++	++++
Normal kidney, serum, and Tyrode's solution in outer chamber.....	—	—
Vaccine virus, frozen kidney, serum, and Tyrode's solution in outer chamber.....	+++++	+++++
Normal kidney, serum, and Tyrode's solution in inner chamber.....	—	—



The results of the experiments just described show (1) that vaccine virus in a mixture of dead cells (killed by freezing and thawing), serum, and Tyrode's solution lost its activity when incubated at 37°C. in test tubes for 4 days; (2) that vaccine virus, in a cell-free preparation of serum and Tyrode's solution placed in a dialyzing apparatus on the opposite side of the membrane from a mixture of normal cells, serum, and Tyrode's solution, retained some but not all of its activity; (3) that vaccine virus, in a mixture of dead cells (killed by freezing and thawing), serum, and Tyrode's solution placed in a dialyzing apparatus on the opposite side of the membrane from a preparation of normal cells, serum, and Tyrode's solution, apparently retained all of its activity. In the light of these findings, it seems that, under the conditions of the experiments, the living cells furnished for the survival of the virus something labile and capable of diffusing through semi-permeable membranes, while the cells killed by freezing and thawing supplied another factor relatively stabile and non-diffusible. However, this explanation of the observed phenomena should not be accepted as correct until it is substantiated by further work.

#### DISCUSSION

Maitland's fluid medium and a simple dialyzing apparatus devised by us made possible investigations concerning certain functions served by living host cells in the survival of vaccine virus at 37°C. Since collodion sacs, through which vaccine virus could not diffuse, were used in the dialyzing apparatuses, it is obvious that the prolonged survival of the virus on one side of the membranes was not dependent upon a direct contact with the living host cells on the opposite side. Therefore, it appears that the living cells furnished something capable of reaching the vaccine virus by diffusion. Inasmuch as a number of substances, even gases in solution (14), can pass through the type of membranes employed, the nature of the diffusible substance supplied by the living cells is not yet known. Experiments, designed to obtain more information concerning the phenomena described in this paper, are now being conducted and the results of these investigations will be reported later.

## SUMMARY

Vaccine virus, suspended in a mixture of serum and Tyrode's solution and separated by collodion membranes from a suspension of living kidney cells in serum and Tyrode's solution, remained active at 37°C. for a longer period of time than did vaccine virus incubated only in a mixture of serum and Tyrode solution.

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# A COMPARISON OF THE METHOD OF EXCRETION OF NEUTRAL RED AND PHENOL RED BY THE MAMMALIAN KIDNEY\*

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During the last decade a considerable addition to our knowledge of kidney function has been made by the study of renal activity in the comparatively simple mesonephroi of the amphibia. The kidney of the frog in particular has been subjected to investigation by a long series of observers who have used methods, such as direct observation (Richards (1)) and perfusion (Höber (2)), to which the mammalian kidney does not readily lend itself. As a result facts have been ascertained directly concerning processes which up to the present time have been investigated in mammals only by indirect methods. These latter methods have of necessity been based largely on hypothesis and have produced chiefly theory, so that if the phenomena observed directly in the frog's kidney could be demonstrated in the mammalian kidney a considerable advance would be made. It has been tacitly assumed by many that an analogy between the two types of animals is a proper one, but since little if any direct evidence has been produced to support such a contention the present study is offered as an attempt at such experimental confirmation.

The possibility of the investigation arose in the following way. In a recent study of the manner of excretion of phenol red and neutral red by the perfused frog's kidney an interesting contrast was found in the mechanism of elimination of the two dyes (Oliver and Shevsky (3)). Phenol red was found to be excreted chiefly through the glomeruli

\* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacology and Chemistry, American Medical Association.

while the more colloidal neutral red was eliminated almost entirely through the tubules. It would seem, therefore, that with such a striking dissimilarity in the manner of excretion of these two dyes by the frog's mesonephroi that experiments on the excretion of the two dyes by mammals, under varying conditions, might cast some light on the question as to the possibility of analogous contrasting mechanisms of elimination in mammalian kidneys.

Another point of interest also presents itself in the study of the excretion of the two dyes by mammals. Phenol red has long been used as a clinical test of kidney function. If it should prove to be the case that neutral red is excreted in a different manner and by a different mechanism by mammals it is possible that it might serve as an adjunct to the phenol red test of the kidney.

With these points in mind the excretion of the two dyes has been studied under various conditions in frogs and rabbits.

### *Frog Experiments*

*Methods.*—In the frog experiments the following methods were used. The kidneys of large *Rana catesbiana* of from 850 to 1000 gms., weight were perfused by the method which we have previously described in detail (3). Very briefly, the perfusion fluid is a modified Locke's solution containing sugar which is led to the kidneys by the renal arteries, thus supplying the glomeruli and at the same time by the renal portal venous system which perfuses the tubular circulation. Pressures of 40 and 20 cm. of water are used on the artery and vein respectively.

Under these conditions the kidney produces a urine which is normal in amount and in its constituents. The volume varies from 7 to 10 ccm. per hour, urea is concentrated, salts are diluted, sugar is retained and colloidal substances such as proteins and gum arabic do not pass into the urine. The two dyes phenol red and neutral red, if added to the perfusion fluid in concentrations of around 15 mgs. per 1000 ccm. of fluid, are concentrated many times and as we have stated are excreted by the glomeruli and tubules respectively.

The analytical methods used in studying the urine were as follows: For sugar, Benedict's qualitative solution, for the dyes a Duboscq colorimeter and for the salts a Christiansen ionometer, the results of this latter determination being expressed as per cents of NaCl.

*Experimental.*—In figure 1 is shown the experimental results of perfusing the frog's kidney with a Locke's solution containing urea and phenol red in concentrations of 20 mg. and 500 mg. per liter re-

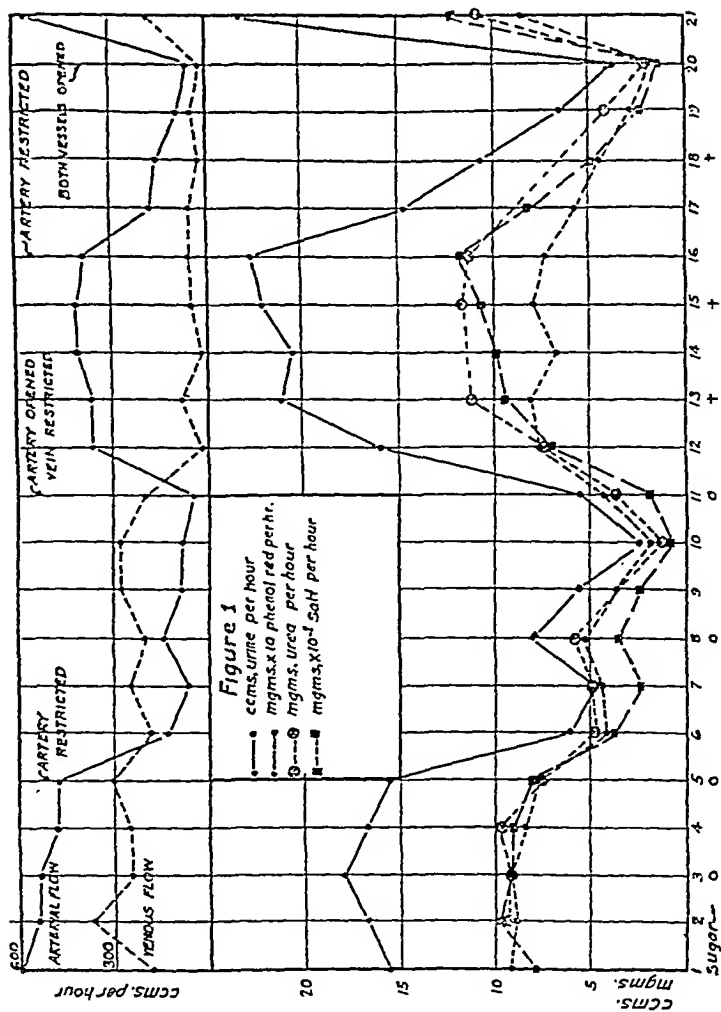


FIG. 1

spectively. These substances were present in the fluid which supplied both the glomeruli and the tubules, so that they were available for excretion by both of these elements of the kidney. In the upper portion of the chart is seen the outflow for the two circulatory systems after the fluid had passed through the kidney.

For the first five periods the perfusion was allowed to proceed in a normal manner. The volume of urine obtained was somewhat higher than that usually obtained, averaging about 16 ccm. per hour but the amount was fairly constant during the entire five periods. No sugar was present in the urine. The concentration ratio, i.e., the concentration of the phenol red in the urine as compared to the concentration in the perfusion fluid was also fairly constant, averaging from a 250 to a 300 per cent increase. The rates of excretion, expressed as mgs. per hour, of urea phenol red and salt were also constant. They averaged 10 mgs. per hour for urea, 1 mg. per hour for phenol red and 100 mgs. per hour for salts.

At the end of the fifth period the flow through the glomeruli was restricted by clamping the rubber tube which leads to the arterial cannula. This resulted in a marked decrease in the arterial outflow, while the circulation through the venous tubular system remained unaffected. Very marked changes were noted at once in the urine. The volume decreased to 6 ccm. per hour, a fall of 62.5 per cent. The rates of excretion of urea, phenol red and salt also fell to approximately one-half their former value.

These conditions continued through the next five periods, during which time the glomerular circulation remained low. The venous circulation remained adequate, however, for at no time was there sugar in the urine.

The interpretation of these findings in the light of our previous findings (3) is plain. A lessened supply of urea, salt, phenol red and water to the glomeruli produced a corresponding decrease in the rate of excretion of these substances and this in spite of the fact that these substances were being administered in excess to tubules which, judged by their ability to absorb sugar, were entirely normal. The major source of excretion of all these substances must have been the same therefore, and this source the glomeruli.

The converse experiment was now performed. At period 11 the

glomerular supply was increased by removing the constriction on the artery and the venous supply to the tubules decreased by restricting the flow to the vein. There was an immediate reestablishment of the former levels of excretion of water, salt, phenol red and urea. In the 13th period the effect of the tubular insufficiency became evident for sugar appeared in the urine, a sign of lack of tubular absorption. For this same reason a moderate "tubular diuresis" resulted, for it will be seen that in periods 13 to 17 although the arterial flow through the glomeruli is less than during the periods of normal perfusion (1-5), nevertheless the rate of water excretion is considerably higher. The same is true of the rates of excretion of urea and salts. In the case of phenol red the previous normal level was not exceeded in this particular experiment.

Under the conditions of this phase of the experiment, therefore, that is with adequate glomeruli supply and with frankly damaged tubules, a result of "anemia" and lack of oxygen, the kidney excreted phenol red, urea and salts at a rate equal to that of the normal kidney. The latter two substances were even excreted at an increased rate, since these substances as has been shown by other methods, are absorbed from the lumen of the tubule when the tubular epithelium is functioning normally.

The remainder of the experiment repeats the previous demonstration of the effect of glomerular "anemia." It will be seen that the rates of excretion of urea, salt and phenol red vary together and follow the rate of excretion of water, and that all these rates depend on an adequate glomerular supply. In contrast to the predominantly glomerular excretion of phenol red figure 2 shows a similar experiment in which the kidneys were perfused with Locke's solution containing urea and neutral red in concentrations of 12.5 and 500 mg. per liter. As in the first experiment both urea and dye were administered to tubules and glomeruli simultaneously by artery and vein and were therefore available for excretion by both of these parts of the renal unit.

In the first period 15 ccm. per hour of urine was excreted. The rate of urea excretion was 15.9 mg. per hour and the rate of neutral red excretion 4.3 mg. per hour. Salts were eliminated at the rate of 48 mgs. per hour. The concentration ratios of the various substances



have not been charted as their variations throughout the experiment are of much less significance than the rates of excretion. During the first period the dye was 23 times as concentrated as the perfusion fluid and the urea three times as concentrated.

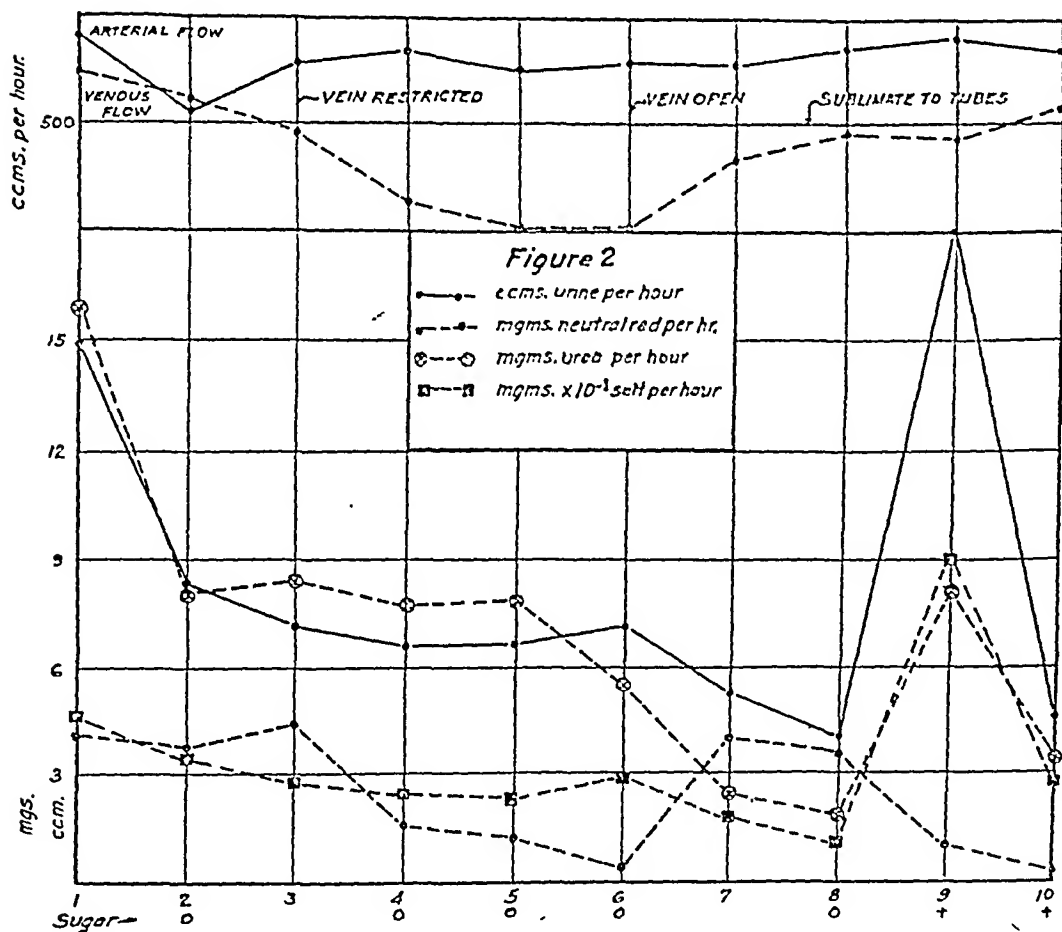


FIG. 2

In period 2 the arterial flow through the glomeruli was lessened and, as a result, the volume of urine excreted fell to 8.6 ccm. per hour. The rate of urea excretion followed this drop to 8.4 mg. per hour, as did the rate of salt excretion (34 mg. per hour), these results being similar in nature to those of the previous experiment. A striking difference is noted in the rate of excretion of the dye. Instead of falling, as did the rate of phenol red excretion in the first experiment,

its rate of excretion was unaffected by the change in glomerular elimination as it remained approximately equal to the previous period. Period 3, under the same conditions, was essentially the same as period 2.

At this point the supply of dye to the tubules was decreased by restricting the flow of perfusion through the vein. In this experiment, however, no actual damage to the tubule cells was produced. No diuresis developed nor did sugar appear in the urine as a result of lack of tubular absorption. The glomerular circulation remained as in the previous periods and the rate of water, urea and salt excretion continued through the next three periods (4, 5 and 6) essentially unchanged. But again the striking contrast in the excretion of the two dyes is seen, as the rate of neutral red elimination instead of following that of urea as did phenol red in the previous experiment, fell progressively to a final figure of .56 mg. per hour.

At the end of period 6 the vein was reopened and the tubules supplied with the former amount of neutral red. Since the tubules, as just noted, had been undamaged in this experiment the rate of neutral red excretion immediately rose to its former figure, 4.1 mg. per hour.

At the end of period 8 a new procedure was introduced into the experiment. 10 ccm. of 1% corrosive sublimate was administered to the tubules during a 10 minute interval at low pressure. We have described elsewhere the result of such a procedure (3). It is followed by all the results of tubule damage, i.e., diuresis and escape of sugar into the urine and, as we will show in a later study, is frequently followed by a repression of urine, probably the result of vascular damage. Our interest here is only in how the dye excretion varies under these unusual conditions.

As will be seen in period 9 a diuresis developed, 18 ccm. per hour, and large amounts of sugar appeared in the urine. As in the previous experiment when the tubules were damaged by "anemia," the rate of urea excretion increased, as did that of salt elimination. But the rate of neutral red excretion instead of accompanying this rise as did phenol red in the first experiment, fell at once and finally reached the extremely low figure of .07 mg. per hour.

In periods 10 and 11 the typical sublimate repression of urine developed and with the fall in water excretion there went the usual fall in rates of urea and salt output.

These two experiments illustrate again the antithesis in the excretion of the two dyes. One, phenol red, behaves as if it were excreted principally by the glomeruli; the other, neutral red, as if the tubules were its chief source of elimination. But for the experiments with mammals which are to follow, the important contribution of these experiments is that the rate of urea excretion may be used as a standard to which the dye excretions may be compared.\* It is by taking advantage of this fact that the following study of the methods of excretion of the two dyes by the mammalian kidney is made possible.

### *Mammalian Experiments*

In following the elimination of dyes and urea by the perfused frog's kidney only the actual rate of excretion of these substances need be followed, for one of the most important factors in determining this rate, namely the concentration in the perfusion fluid, is constant. In the experiments on living rabbits designed to determine whether or not the methods of excretion shown to exist in the amphibian kidney can be transferred to the mammalian kidney, the maintenance of such a state is impossible, since the perfusion fluid here is the plasma of the circulating blood. Experience with the excretion of other substances, such as chloride (4), phosphate (5, 6), creatinine (7, 8) and particularly urea (9), has shown that in the absence of definite knowledge of the behavior of a substance that not the rate of excretion alone but this rate in relation to the plasma concentration or the

excretory ratio:  $\frac{\text{Urine rate}}{\text{Plasma concentration}}$  should form the basis of study

and comparison. In the choice of conditions under which to observe this ratio experience gained in the study of urea excretion was again called upon. The rate of urea excretion by the kidney is influenced by a variety of factors other than the blood urea concentration. Constancy of these factors is best obtained (10) when there is a marked stimulation of renal tissue or when as Addis has pointed out (11) the

\* We do not wish to discuss at this time the mechanism of urea excretion, reserving this for a later communication. In these experiments its method of excretion resembles that of phenol red and its chief source of elimination is the glomerulus.

conditions are such that it seems reasonable to assume that all of the renal elements have been awakened to activity. It seemed highly probable that other factors than the dye concentration of the plasma may accelerate or inhibit phenol red and neutral red excretion and, in that case, it is to be expected that a balance of these factors would also best be obtained during a heightened renal activity. This was attained by the administration of urea and large quantities of water. During the ensuing diuresis simultaneous observations of the serum dye concentration and the rate of dye excretion in the urine were made.

*Methods.*—Healthy male rabbits were chosen for these experiments. When observations were terminated the animals were killed with ether and whenever the kidneys were not entirely normal the experiment was discarded. Except for the low urine volume experiments the general procedure was identical for every animal. No food was given for fifteen hours before the experiment commenced. Three hours before the first catheterization, when observations were begun, 40 ccm. per kilo body weight of a 5 per cent solution of urea was given by stomach tube and every hour thereafter until the experiment was ended 40 ccm. of tap water per kilo was administered in the same manner. The urine was obtained by catheter at approximate intervals as recorded in each experiment. After each catheterization the bladder was thoroughly washed with a known volume of distilled water. Arterial blood was obtained from the heart without an anticoagulant at the middle of each period of urine collection. Analyses were performed on the serum. There is scarcely a demonstrable difference in the urea or dye concentration of serum and paraffine plasma from the same sample of blood. Urea in both the serum and urine was determined by a urease and aeration method. Phenol red and neutral red in the serum and plasma were determined by the addition of NaOH in one case and HCL in the other and comparison after the necessary dilution with known standards in a Dubosq colorimeter. The results are all expressed as milligrams excreted per hour and milligrams per 100 ccm. of serum.

### *Comparison of the Excretion of Phenol Red and Neutral Red*

Numerous experiments were performed with varying degrees of success in comparing the excretion of phenol red with neutral red. The phenol red experiments were usually successful while with the more colloidal neutral red difficulties were encountered. At the normal pH of rabbit plasma this dye in comparison with phenol red is relatively insoluble and in attempting to obtain higher and higher plasma concentrations some animals were killed in so-called "ana-

phylactoid" shock. Whatever the quantity of dye injected by far the greater part rapidly leaves the blood stream and as compared with phenol red only relatively low plasma concentrations can be

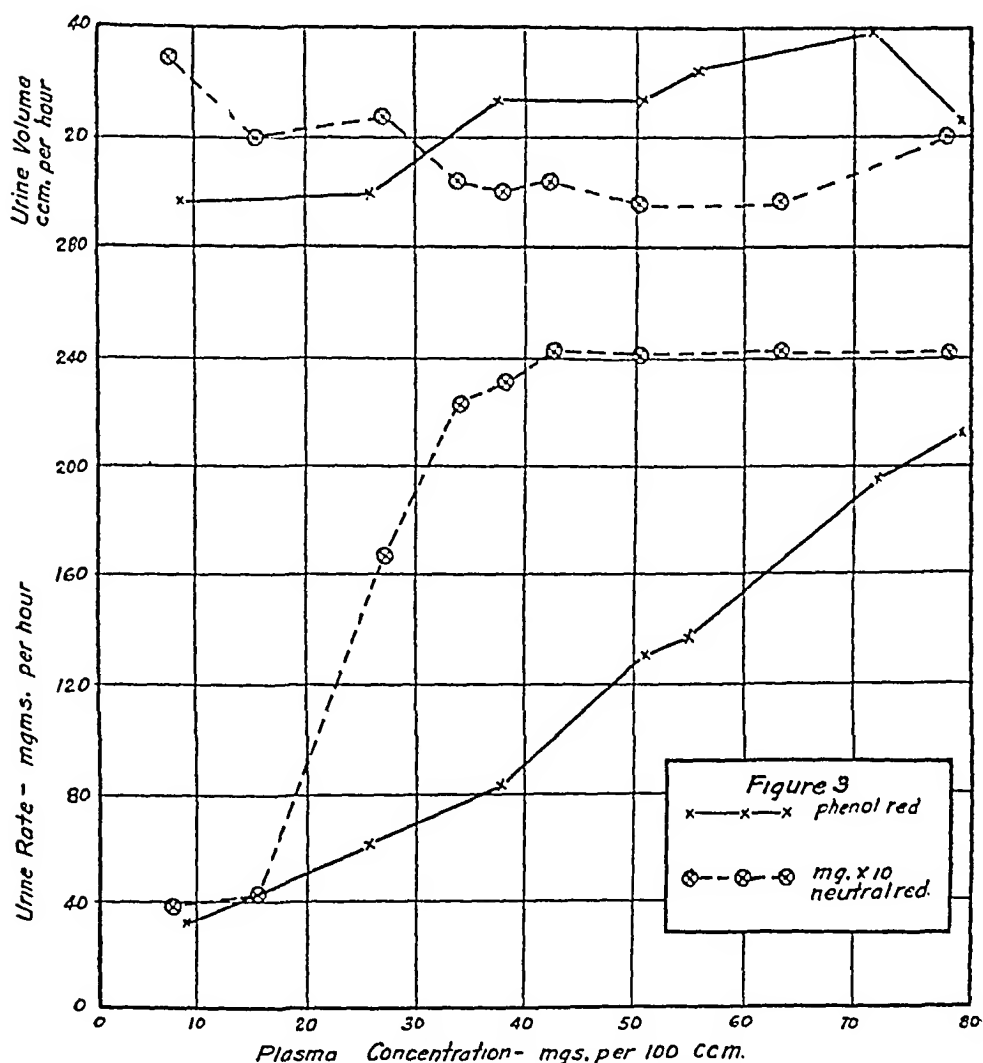


FIG. 3

obtained. Three or more observations were obtained for both dyes in 14 rabbits. In every case the main results were the same. A typical experiment follows:

A male rabbit weighing 2500 grams was given water and urea as described and 3 hours later 500 mgms. of phenol red were given intravenously in 2 per cent

solution. Ten minutes after this the bladder was washed out and the first urine collection commenced. Urine was collected at half hour intervals and serum specimens obtained at the middle of these periods. The observations are plotted in figure 3. Six days later this rabbit was again given urea and water. Thirty minutes before the first urine collection was commenced 100 mgms. of neutral red in 1 per cent solution were injected intravenously. Fifty mgms. of the dye were given every 30 minutes thereafter until the end of the experiment. Urine collections were made at half hour intervals and serum specimens obtained as before.

In figure 3 the neutral red observations have been compared with the phenol red excretion figures. There can be little question that neutral red is excreted in a manner very different from that of phenol red by the mammalian as well as the amphibian kidney. The rate of phenol red excretion in the urine is directly proportional to the plasma dye concentration while the rate of neutral red excretion bears no constant relation to the concentration of neutral red in the plasma except that a maximum rate of excretion independent of the plasma dye concentration is reached while the latter is still at a low figure.

An experiment reported by Marshall and Crane (12) which with other data led these authors to conclude that phenol red was secreted by the tubules of both the amphibian and mammalian kidneys requires some comment at this point. In comparing the rate of phenol red excretion to the plasma phenol red concentration in a dog these investigators failed to find the direct relationship which we have described but instead obtained a curve resembling our own neutral red observations. An examination of the protocol of their experiment reveals possible reasons for the discrepancy between their results and ours. They collected urine specimens as small as 1.0 cc. and even with the ureters cannulated these volumes are too small to give any degree of accuracy to urine collections from a dog's kidney when the tubule and pelvis dead space is taken into consideration. Another factor which would nullify their unsupported observations is the time interval used for urine collections. In no instance did this exceed the very short period of 3 minutes. Another and perhaps the most important factor contributing to the nature of their results is the fact that blood samples were taken not at the middle but at the beginning of the 3 minute periods of urine collection. Presumably this was done to correct for the urine volume of the kidney dead space and would conceivably have done this had the urine volume remained the same throughout the observations. This however was not the case and the deviations from a direct plasma concentration—urine rate relationships are in a general way those which might be expected from the variation in the urine volume.

*Comparison of the Appearance Time of Phenol Red and Neutral Red*

Another marked difference in the excretion of phenol red and neutral red which is not shown in the type of experiment illustrated in figure 3 is the interval between the commencement of the intravenous injection of the dye solution and the time of its appearance in the bladder urine. In comparison with phenol red neutral red was always very slow in making its appearance in the urine. By continuous washing of the bladder during a marked diuresis the dye appearance time was determined with a fair degree of accuracy in a number of experiments. In the experiments where it was recorded neutral red appeared in the bladder urine 3, 5, 3, 9, 12, 15, 16, 16, 17 and 22 minutes after the intravenous injection was begun. The appearance time apparently bore no relation to the dose. In one case phenol red required over 4 minutes to reach the urine but in more than a dozen other experiments the appearance time was uniformly less than 1 minute. If we assume an analogy between the excretory mechanisms of the frog and rabbit kidneys in so far as these dyes are concerned it is easy to visualize the reason for the marked difference in the appearance time of these two dyes. Phenol red would pass through in the glomerular filtrate almost immediately on reaching a *fully active* kidney and would soon be seen in the urine. On the other hand it would seem reasonable that neutral red, if secreted by the tubules, requires a measurable length of time to be removed from the plasma into the cells and passed into the urine in any amount.

*Comparison of Phenol Red and Urea Excretion*

Phenol red and urea are excreted in a similar manner by the perfused frog's kidney. Proof that this was also true in the rabbit would contribute additional evidence indicating an analogy in the mechanisms of renal function of these two types of animals. The following experiment is typical of a group of five in which excretion of phenol red and urea were simultaneously compared.

A male rabbit weighing 2.5 kilos was treated as previously described and 15 minutes before the collection of urine was begun the animal received 15 cc. of 5 per cent phenol red solution intravenously. Urine was collected over eight

30-minute periods and blood samples taken at the mid-point of each of these periods. The results form figure 4.

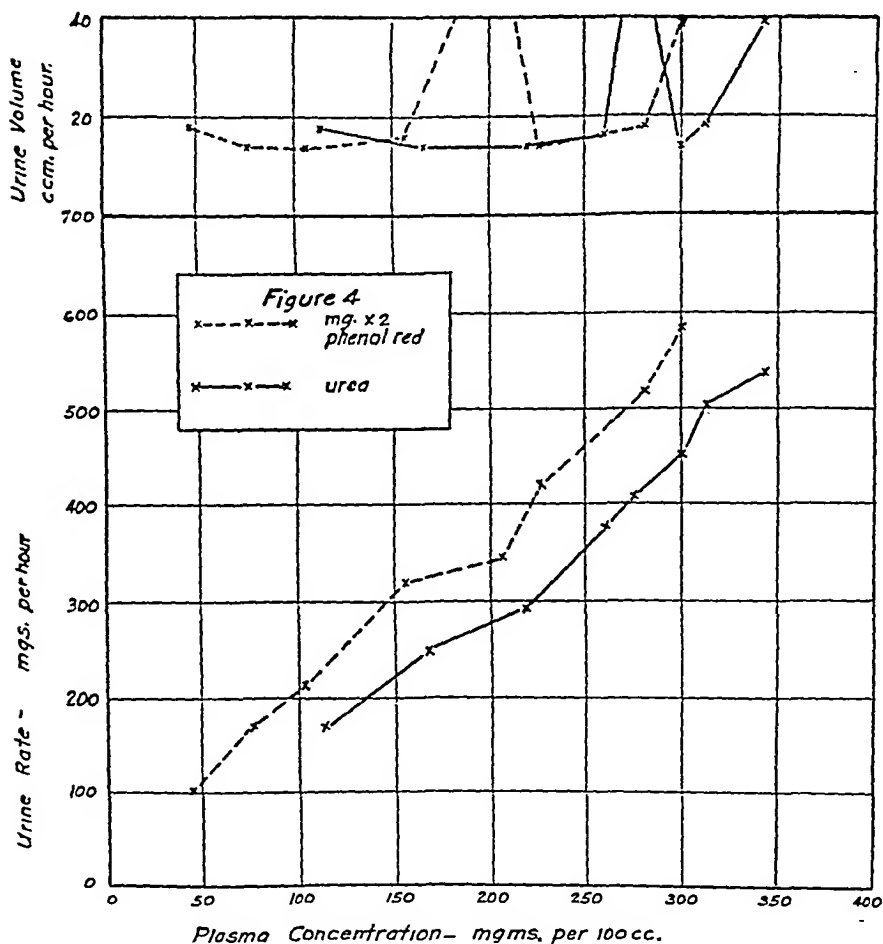


FIG. 4

Phenol red then is excreted by the mammalian kidney in the same way as urea, that is, in both cases the rate of excretion in the urine is directly proportional to the plasma concentration. In the particular experiment cited here the ratios:  $\frac{\text{Urine Rate}}{\text{Plasma Concentration}}$  are rather small for both urea and phenol red in relation to the size of the rabbit



and at postmortem the kidneys were found to be normal but considerably smaller than usual. The difference between the ratios, that of phenol red being higher than that of urea, is a constant finding which will be discussed in another communication.

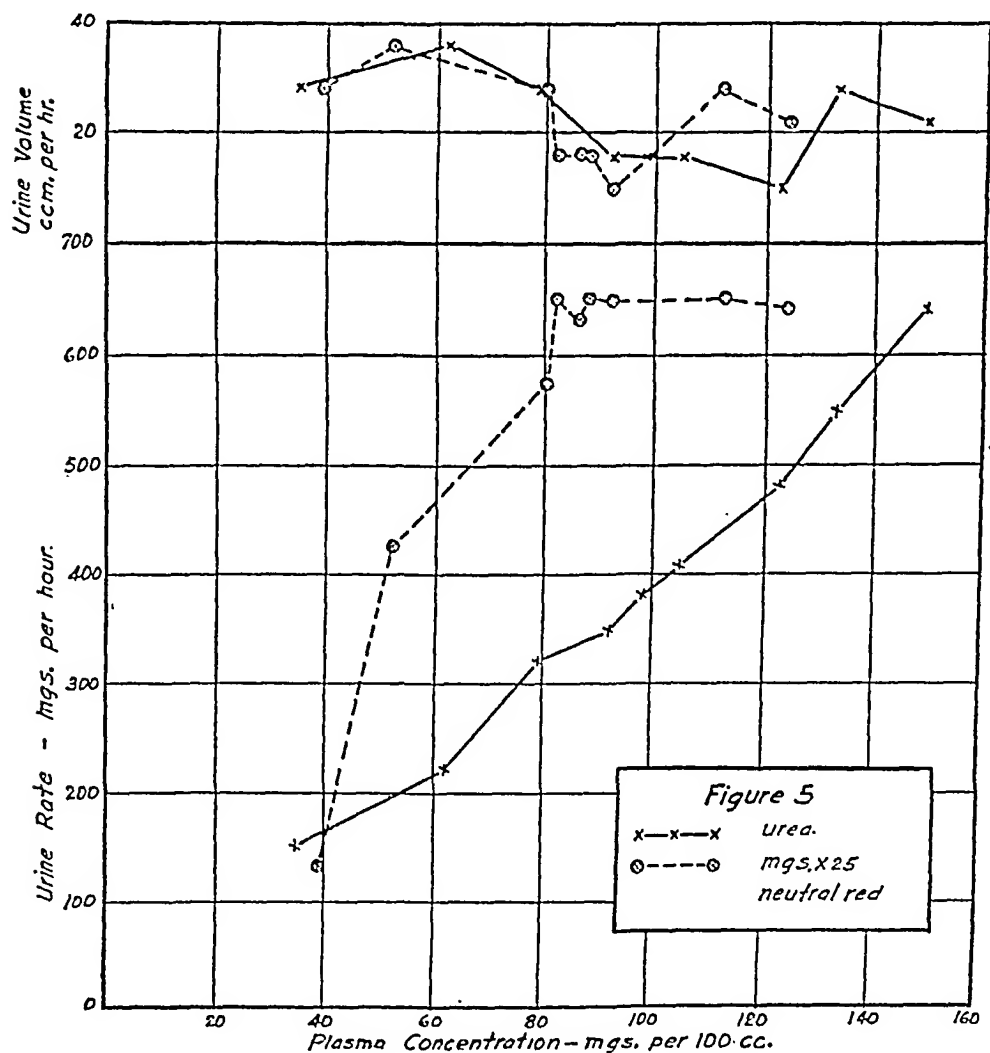


FIG. 5

### *Comparison of Neutral Red and Urea Excretion*

In contradistinction to phenol red, neutral red is excreted by the amphibian kidney in a manner unlike urea. Likewise neutral red

and urea are excreted differently by the mammalian kidney. A typical experiment, one of three in which this point is demonstrated, follows:

A 3.5 kilo male rabbit received water and urea as described previously. Neutral red was given intravenously as in experiment 3, 175 mgms. being injected in 1 per cent solution each half hour. Nine 30-minute urine collections were made, a sample of serum being obtained at the middle of each one.

The results in figure 5 show the usual relationship under these conditions for urea, the urine rate being directly proportional to the plasma concentration. The excretion of neutral red on the other hand bears no consistent relation to the plasma concentration at low levels of the latter and at higher levels the rate remains constant despite further increases in the concentration of dye in the plasma.

#### *Relation of Phenol Red Excretion to Urine Volume*

Rowntree and Geraghty (13) and other observers (Marshall and Kolls (14)) have held that the rate of phenol red elimination by the kidneys is independent of the fluid output. If this were so the excretion of this dye would be different in this respect from urea. Although no direct relationship between the rate of urea excretion and the urine volume has been demonstrated it has been shown (15, 16) that both the rate of urea excretion as measured by the ratio:

$$\frac{\text{Urine Urea Rate}}{\text{Blood Urea Concentration}}$$
 and the urine volume tend to vary with the degree of renal activity. Low urine volumes and low urea ratios are generally found at low degrees of renal activity and increase with the degree of renal activity until the urea ratio is at a maximum when the urine volume alone may continue to increase. If phenol red and urea are excreted by the same mechanism the relationship of the excretory ratios to the urine volume should be similar. That this is so in the frog's kidney has been shown in the experiments already described and the experiment which forms figure 6 demonstrates that this is also the case in the mammalian kidney. These observations were obtained as follows:

A 3.5 kilo male rabbit was kept without food or water for 15 hours. At the end of this period 3 ccm. of a 5 per cent phenol red solution were injected intra-

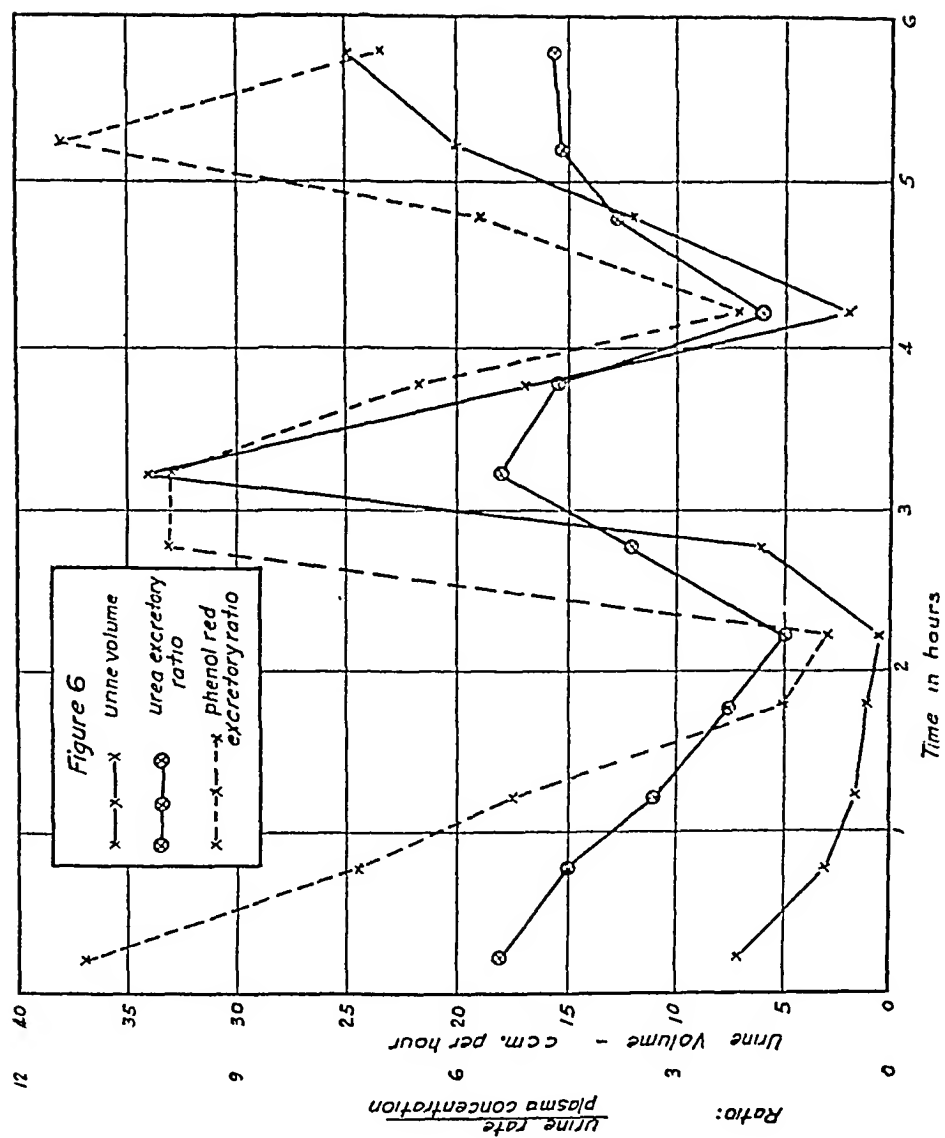


FIG. 6

venously and ten minutes later the bladder was drained and washed with several small known volumes of distilled water all of which was returned. During the ensuing 6 hours 12 half-hour urine collections were made, the bladder being washed out at the end of each one with four 10 cc. portions of water. A sample of blood was obtained at the middle of each urine period. One and a half hours after the first urine collection was commenced 3 cc. more of 5 per cent phenol red solution were given by ear vein. At 2.5 hours 100 cc. of distilled water were given by mouth. Five cc. of 2 per cent urea and 2 per cent phenol red were given intravenously at 3 hours and 10 cc. at 4.5 hours. At 5 hours the rabbit received 10 cc. of 20 per cent creatinine and at 5.5 hours 15 cc. of 20 per cent creatinine and 0.2 per cent phenol red by intravenous injection.

The observations in this experiment show very clearly that the rate of phenol red excretion, just as does the rate of urea excretion, fluctuates with the urine volume under conditions of less than full renal activity. This result is at variance with the view which is generally held that no changes in urine volume are associated with changes in phenol red excretion. The reason for this is that previous observers have neglected to take into consideration the plasma dye concentration and have dealt not with the rate of excretion but with the percentage of an injected quantity which appeared in the urine in an arbitrary period.

#### SUMMARY

A direct examination with the method of perfusion of the excretion by the frog's kidney of phenol red and neutral red has shown that the dyes are eliminated in different manners as a result of different mechanisms. The former is excreted in much the greater part by the glomeruli; the latter by the tubules. Urea is excreted in a manner similar to phenol red.

The indirect examination of the function of the mammalian kidney by means of excretion ratios has shown a like contrast between the manner of elimination of the two dyes, and here again was found a similarity in the manner of excretion of phenol red and urea.

This would seem to be as close an examination as can be made with our present methods of experimentation of the question of the mechanism of the excretion of these substances by mammals. As the facts stand they constitute strong presumptive evidence that in mammals and amphibia the like results have arisen from like causes,

phenol red and urea being eliminated chiefly through the glomeruli in both instances while neutral red is excreted principally through the tubules.

### CONCLUSIONS

1. There is a difference in the manner of excretion of phenol red and urea from that of neutral red by the frog's kidney.

2. This difference is due to differences in mechanism of excretion, the elimination of the former being by the glomeruli and the latter by the tubules.

3. There is a similar difference in the manner of excretion of these substances by the mammalian kidney.

4. It is inferred that this similar difference in manner of excretion is due to a similar difference in method of elimination, and that in mammals too, phenol red is eliminated chiefly through the glomeruli and neutral red through the tubules.

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# VARIATIONS IN THE PLASMA CHOLESTEROL AND CHOLESTEROL ESTER CONTENT IN HOG CHOLERA

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Abnormalities in the cholesterol content of the blood have been noted in a number of acute infectious diseases.

Bacmeister and Henes (1) observed that during the stage of hyperpyrexia in acute infectious diseases as e.g. typhoid fever, scarlet fever, pneumonia, erysipelas and sepsis the blood cholesterol content was less than normal. With defervescence the blood cholesterol content approached or rose somewhat above its normal level. On the other hand, Gorter and ten Bokkel Hinnink (2) found that infection of rabbits with a virulent strain of paratyphoid bacilli resulted in a considerable hypercholesterolemia in those cases in which infection was rapidly fatal. In less acute infections, which did not terminate fatally, the hypercholesterolemia gradually disappeared and the cholesterol content became normal without having exhibited subnormal values. Kipp (3) found that in pneumonia the changes in the serum cholesterol content could be divided into three stages; first, a hypocholesterolemia, early in the disease, the degree of which was dependent upon the amount of lung involvement and the intensity of the infection; secondly, a hypercholesterolemia during and continuing for a varying period of time after the resolution of the pneumonic exudate, and lastly, a return to normal with convalescence. Stern (4) observed that in scarlet fever the hypocholesterolemia present at the onset of the infection gradually changed until the values reached the lower limits of normal, where they persisted throughout the latter part of the infection and during convalescence. Malinut and Santner (5) found that in puerperal fever the blood cholesterol level was increased even above the hypercholesterolemia of pregnancy. De Paula Santos (6) and Borel, Pons, Advier, and Guillermin (7) observed that in malaria the cholesterol content of the blood was subnormal. A hypocholesterolemia was noted by Rouzaud, Sucquet, and Cabanis (8) in the secondary stage of syphilis, normal values being found in the primary stage. Marie (9) noted that rabies was ordinarily accompanied by a hypercholesterolemia.

In general, then, most bacterial diseases thus far studied show subnormal blood cholesterol values, paratyphoid infection in rabbits and

puerperal sepsis being apparent exceptions. In the case of the latter as in rabies, a filterable virus disease, there is a hypercholesterolemia. The mechanism of or functions served by the changes observed in the blood cholesterol have never been adequately explained. Some suggestions have, however, been advanced by various investigators.

Leupold and Bogendörfer (12) considered that the hypocholesterolemia observed during acute infections was the result of combination of cholesterol with toxic products of the infectious agent in a protective manner. That such a combination is conceivable is indicated by Ransom's (13) investigations concerning the inhibitory action of cholesterol for saponin hemolysis and Noguchi's (14) concerning its antihemolytic activity against agaricin and tetanolysin. Numerous subsequent investigators have observed other toxic substances that could be neutralized in whole or in part by cholesterol *in vitro*. *In vivo*, the antitoxic action of cholesterol is less evident. Filia (15) who noted that cholesterol, *in vitro*, had a neutralizing action on diphtheria toxin observed that it was completely devoid of action when injected into an animal given a fatal dose of diphtheria toxin. Lubinski and Stern (16) were unable to protect mice and rabbits against dysentery, tetanus or diphtheria toxins by the administration of considerable amounts of cholesterol. Beumer (17) noted that guinea pigs fed large amounts of cholesterol lived longer than controls when given a lethal dose of diphtheria toxin.

The effects on the blood cholesterol content of the injection of toxic substances into animals have been observed. Hueck (18) reported for Köhler that the injection of saponin into animals caused, at first, a reduction in blood cholesterol, followed by an increase. By repeated small doses the blood cholesterol could be maintained above its normal level. Kollert, Kofler, and Susani (19) and Handovsky and Trössel (20) noted only an increase following the administration of saponin to animals. Adler (21) and Yonemura and Fujihara (22) found that intravenously injected bile acids reduced the cholesterol content of the blood. The latter investigators called attention to the fact that certain snake venoms had a similar effect on the blood cholesterol. Leupold and Seisser (23) used, as one of their methods for lowering the blood cholesterol of rabbits, the injection of diphtheria toxin.

Recently experimental work on the filterable virus disease, hog cholera, has been conducted in this laboratory and it seemed of interest to determine the variations in plasma cholesterol and cholesterol ester associated with it. Most of the previous investigations bearing on changes in the blood cholesterol content in acute infectious diseases have had to do with spontaneous disease in human subjects and hence have not been entirely controllable or as complete as is possible with induced infections in animals.

## EXPERIMENTAL

Inoculation with hog cholera virus was made either intraperitoneally, intracutaneously, or intracorneally. Blood upon which the normal plasma cholesterol and cholesterol ester values were to be determined was drawn either the day the animals were inoculated with virus, just preceding this inoculation, or on the preceding day. The animals were bled from the artery of the tail into a tube, and heparin, 1 mg. per 5 cc. of blood, was used as the anticoagulant. Bloor's method (10) was used in determining the total plasma cholesterol and Bloor and Knudson's method (11) in determining plasma cholesterol ester.

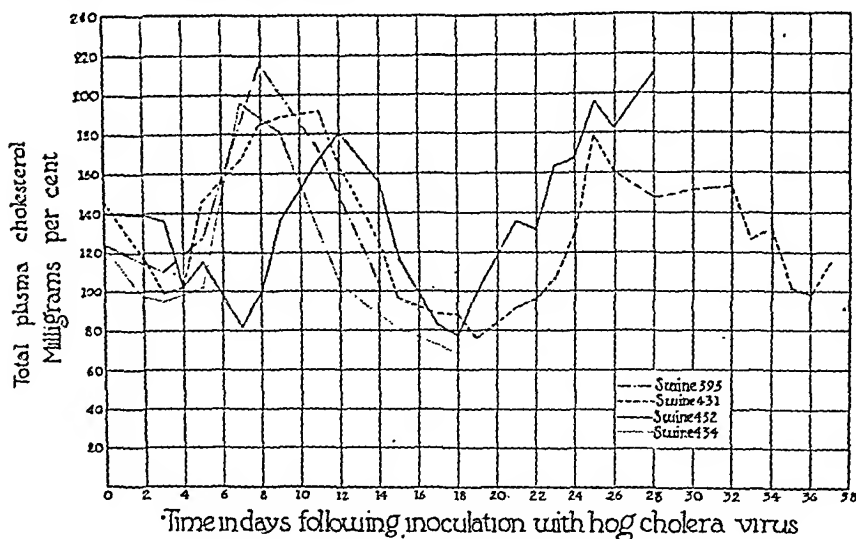


CHART 1

## RESULTS

The findings, as regards changes in plasma cholesterol and cholesterol ester, in hog cholera are summarized in Table I and represented graphically in Chart 1. The variations observed were quite uniform for the group of animals as a whole, only slight individual differences being noted. Immediately following inoculation with virus the plasma cholesterol and cholesterol ester content diminished, the hypocholesterolemia persisting in all cases for at least 3 days following inoculation, and in the cases of Swine 432 and 434 for somewhat longer. This interval corresponded to the "incubation" period of the disease and terminated soon after the febrile reaction to the infection

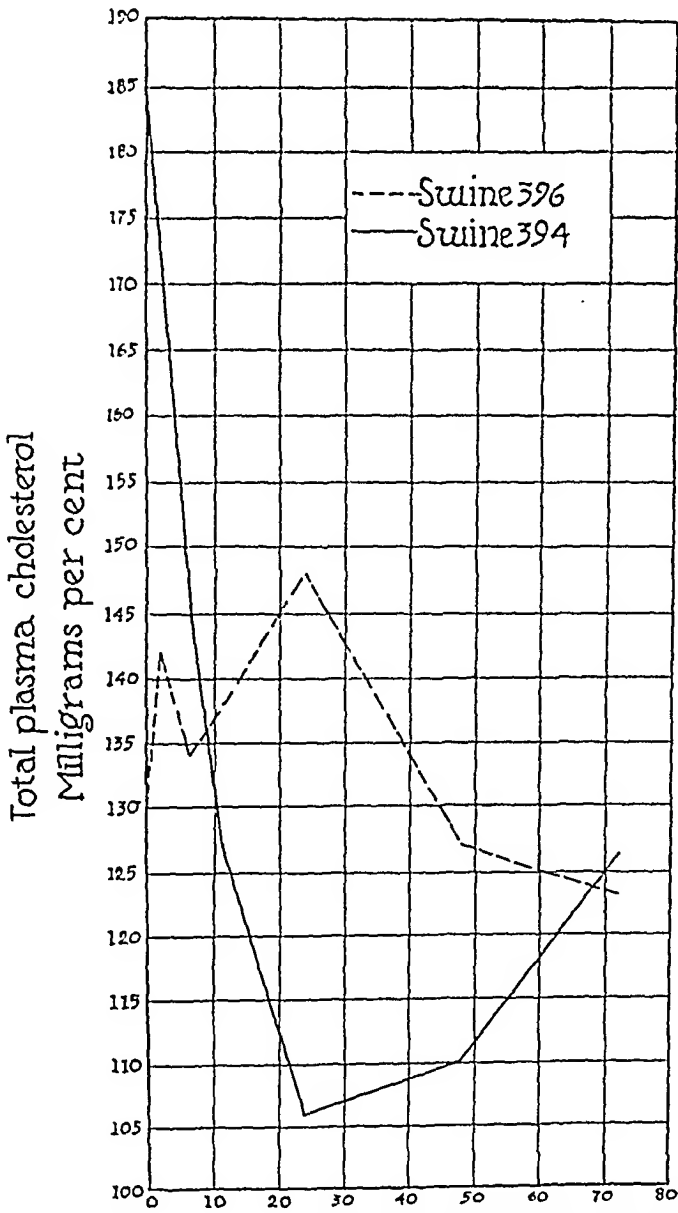


TABLE I

Days after inoculation	Swine 393				Swine 431				Swine 432				Swine 434			
	Temperature	Total plasma cholesterol	Plasma cholesterol ester		Temperature	Total plasma cholesterol	Plasma cholesterol ester		Temperature	Total plasma cholesterol	Plasma cholesterol ester		Temperature	Total plasma cholesterol	Plasma cholesterol ester	
	°C.	mg. per cent	mg. per cent	per cent of total	°C.	mg. per cent	mg. per cent	per cent of total	°C.	mg. per cent	mg. per cent	per cent of total	°C.	mg. per cent	mg. per cent	per cent of total
Normal	38.7	124	70	56	39.0	146	78	53	39.0	140	85	61	38.6	123	74	60
	0.001 cc. hog cholera virus intraperitoneally				0.4 cc. hog cholera virus intracutaneously				0.1 cc. hog cholera virus intracorneally				0.4 cc. hog cholera virus intracutaneously			
2					39.2	117	55	47	39.4	139	92	66	38.9	97	74	76
3	40.4	110	54	49	40.7	99	53	54	39.9	136	88	65	39.5	95	72	76
4					41.5	102	57	56	39.5	102	87	85				
5	41.0	127	83	65	41.6	146	63	43	39.8	115	68	59	40.9	102	65	64
7					41.5	167	92	55	41.8	82	47	57	41.4	196	103	53
8	41.6	216	157	73	42.0	185	104	56	42.0	100	54	54				
9					42.1	189	113	60	41.2	136	69	51	41.6	181	98	54
11	41.0	172	113	66	41.6	192	126	66	42.8	167	134	80	41.2	130	90	69
12					41.0	164	95	58	42.2	181	144	80	41.6	104	61	59
14	40.1	105	57	54	40.4	126	65	52	40.5	156	107	69				
15					40.3	97	57	59	40.1	117	80	68	41.0	81	42	52
17					39.5	89	50	56	39.0	83	50	60				
18					39.5	89	51	57	38.5	78	50	64	40.7	69	35	51
19					38.5	77	54	70	38.6	100	48	48				
21					38.4	92	61	66	37.0	136	72	53				
22					38.8	96	57	58	37.0	132	79	60				
23					39.2	107	73	68	35.8	164	59	36				
24					38.8	130	75	58	36.8	168	51	30				
25					39.2	179	86	48	36.2	196	43	22				
26					38.2	160	93	58	39.5	183	56	31				
28					39.0	148	79	53	38.9	211	92	44				
30					39.3	152	78	51								
31					40.0	153	94	61								
32					39.3	154	83	54								
33					39.6	127	92	72								
34					39.5	132	86	65								
35					40.1	102	89	87								
36					40.1	99	63	64								
37					39.5	115	69	60								

became established. Following the early period of hypocholesterolemia a stage of rather marked hypercholesterolemia ensued. This period was coincident with the onset of fever, general malaise, irritability, and loss of appetite. The period of hypercholesterolemia was succeeded by a second stage of hypocholesterolemia more marked as to degree and more prolonged than that during the period immediately following inoculation had been. During this stage all of the general symptoms of the disease had become more marked. Physical depression was as a rule complete, the animals lying in their pens in a torpid condition and not moving unless disturbed. Food was completely refused and very little water was taken. There was marked weakness of the legs; loss of weight was evident; respiration was more rapid and noisy; purple splotching of the skin was appearing; and the fever was beginning to diminish. While in this condition Swine 393 and 434 became moribund and were killed for pathological material. Swine 396 was killed for pathological material before it reached this stage. Swine 431 and 432, animals with a less acute type of cholera than the others, showed a second period of hypercholesterolemia. This persisted in Swine 432 until its death and in Swine 431 was maintained for over a week and then was succeeded by a third period of a rather fluctuating hypocholesterolemia. The animals during the last period of hypercholesterolemia had become markedly emaciated, were delirious, unable to stand, with incoordinated movements and a beginning dry gangrene of the ears and areas of the skin. Swine 431 was killed when in this condition for pathological material.

The changes and fluctuations in the plasma cholesterol content in hog cholera were so constant that it seemed of interest to determine the effect of a secondary infection. For this purpose, *Bacillus suisep-ticus* was chosen since it had been observed that inoculation with this organism was followed very promptly by a marked decrease in the plasma cholesterol content. Swine 396 was inoculated with hog cholera virus and the disease allowed to progress for 7 days through the stage of hypocholesterolemia and into the beginning of the stage of hypercholesterolemia. Then this animal and a normal one, Swine 394, were inoculated subcutaneously with the same culture of *B. suisep-ticus*. The variations in plasma cholesterol and cholesterol



Time in hours following the subcutaneous inoculation of 5cc. of a 24 hour broth culture of *B. suis* septicus (376D)

CHART 2

ester observed are given in Table II and represented graphically in Chart 2. The reaction of the cholera-infected animal to infection with *B. suis*epiteticus was very unlike that of the normal healthy animal. The latter showed a marked and rapidly progressive decrease in plasma cholesterol, the content reaching a low point in 24 hours. The cholera-infected animal, on the other hand, failed completely to show any decrease in plasma cholesterol during the corresponding 24 hour

TABLE II

Time after inoculation		Swine 396				Time after inoculation		Swine 394			
		Tem- pera- ture	Total plasma choles- terol	Plasma choles- terol ester				Tem- pera- ture	Total plasma choles- terol	Plasma choles- terol ester	
days	hrs.	°C.	mg. per cent	mg. per cent	per cent	days	hrs.	°C.	mg. per cent	mg. per cent	per cent
Normal		39.2	122	79	65						
0.1 cc. hog cholera virus intraperitoneally											
1		39.4	104	76	73						
2		40.2	111	78	70						
3		41.0	108	28	26						
7		42.0	131	87	66						
						Normal		39.2	185	91	49
5 cc. of a 24 hour broth culture of <i>B. suis</i> epiteticus (376D) subcutaneously											
	2	42.2	142	87	61						
	6½	41.6	134	92	69		6½	41.6	144	76	53
	11½		138	83	60		11½	41.4	127	75	59
8		41.1	148	83	56	1		39.5	106	70	66
9		42.0	127	68	54	2		39.0	110	74	67
10		41.8	123	68	55	3		39.0	126	79	63

period. Instead a slight increase occurred which may or may not have been a part of the regular cholera hypercholesterolemia. The plasma cholesterol content of Swine 394 began to return towards the normal sometime after the first 24 hours whereas in Swine 396 the initial slight hypercholesterolemia decreased slightly. Altogether the changes in plasma cholesterol following inoculation with *B. suis*epiteticus were entirely unlike those taking place in the normal animal.

## DISCUSSION

The constancy of the changes in plasma cholesterol and cholesterol ester observed throughout the course of experimentally induced hog cholera suggests a uniformity of mechanism by which the variations are effected. The finding that there is an initial stage of hypocholesterolemia agrees well with observations made by earlier investigators on other acute infectious diseases. The fluctuating plasma cholesterol alterations in hog cholera are unique, however, similar observations having been made in no other acute infection. The significance of these fluctuations is not known. The assumption of previous investigators that in acute infectious diseases variations in blood cholesterol are a manifestation of an antitoxic process, could at most only partially explain the fluctuations observed in hog cholera. These fluctuations may be only an indication of derangement of the physiological mechanism whereby the blood cholesterol level is controlled and be devoid of significance for the clinical course of the disease. The failure of a cholera-infected animal to show a decrease in plasma cholesterol content when inoculated with an organism (*B. suis*septicus) known to be capable of inducing a marked decrease in a normal animal, if it proves to be a regular finding, might be evidence of an inability of the cholesterol regulatory mechanism to function in swine ill with hog cholera.

## SUMMARY AND CONCLUSIONS

1. The plasma cholesterol and cholesterol ester content of swine, experimentally infected with hog cholera, exhibit a regular succession of changes. During the period of incubation of the disease, for 3 or more days following inoculation with hog cholera virus, hypocholesterolemia prevails. This is followed by a period of hypercholesterolemia which is coincident with the onset of the clinical manifestations of the disease. The hypercholesterolemia after persisting for from 4 to 7 days, gives way to a second period of hypocholesterolemia more marked and more prolonged than that observed immediately after inoculation. In the experiments of the present work this second period lasted 8 and 11 days in the 2 animals surviving long enough for the study of it and was followed by a second period of

hypercholesterolemia. In the one animal surviving this period for 8 days a third period of irregular and fluctuating hypocholesterolemia set in.

2. A comparison with the results in other acute infections indicates that hog cholera is unique in showing alternating periods of hypocholesterolemia and hypercholesterolemia.

3. A normal hog inoculated with *Bacillus suissepticus* rapidly developed the typical marked hypocholesterolemia whereas an animal infected with hog cholera and then inoculated with *B. suissepticus* failed to show the decrease in plasma cholesterol content.

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# ON THE RELATION OF THE ORGANISMS IN THE TUNICA VAGINALIS OF ANIMALS INOCULATED WITH MEXICAN TYPHUS TO RICKETTSIA PROWAZEKI AND TO THE CAUSATIVE AGENT OF THAT DISEASE

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## PLATE 5

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### INTRODUCTION

In earlier communications (1, 2) one of the present authors has advanced considerable evidence that the small intracellular organism discovered by him in the tunica vaginalis of guinea pigs reacting to the virus of tabardillo represents the specific organism of Mexican typhus. This organism can easily be demonstrated in all animals killed during the early scrotal reaction, whereas it never was met with in numerous normal control animals nor in animals which had recovered from the experimental disease. In its morphology, staining properties and its mode of intracellular multiplication, it exhibits a striking resemblance to *Rickettsia prowazeki* as found in the gut of lice fed upon typhus patients. The tunica vaginalis regularly proved to be the most infectious tissue, more so than brain, and a positive Weil-Felix reaction can be induced in rats and in rabbits more regularly with tunica than with blood or brain. The same small micro-organism was recently also demonstrated in both of these species. The rat proved to be an even more appropriate animal for the demonstration of this micro-organism than the guinea pig, as was first shown by Maxcy (3). It is frequently found in smears from the tunica of white rats in astonishing numbers within greatly swollen cells from the endothelial covering of the visceral and parietal surfaces. The relation of this organism to typhus fever has been so far cleared up that we were able



to state that it could not be separated etiologically from our strain of Mexican typhus. We still lacked, however, some very important evidence for the identity of the two, namely, the demonstration of the tunica organism in animals inoculated with lice infected with the virus of our strain. Although the findings of Maxcy in a strain of endemic typhus from North Carolina (4) and those of Pinkerton in a strain from Europe (5) have brought full confirmation of the findings of Mooser, the possibility that the tunica organism is a mere secondary invader had not yet been completely excluded. When one considers that the blood serum of a typhus patient regularly agglutinates in high dilutions *B. proteus* X19 and frequently the bacillus of Plotz, both of which have no etiological relation to that disease, even an organism found with such regularity as the tunica germ must be looked upon with suspicion. Progress can only be expected from experiments with infected lice. The present paper deals with this problem.

### *Material and Methods*

1. Three monkeys have been used in these experiments, Monkeys 1, 2, and 3. As these three animals have not been inoculated at the same time and with the same material, the account of their reaction after inoculation and the result of lice feeding experiments will be presented separately.

2. The strain of body lice used in these experiments was carefully examined for Rickettsia-like organisms before we started. No suspicious organisms could be found in smears from very numerous specimens. The intestinal tract of the great majority proved to be free from demonstrable micro-organisms. The same was true for a number of lice fed for a week on a normal monkey. As the lice did not feed when applied to the monkeys within a Nuttall box, the felt with the lice on it was removed from the boxes each time for feeding and applied directly to the shaved abdomen. They were always closely watched for the whole time of feeding. As a rule they were applied twice a day to an infected monkey and, between feedings, they were kept in an incubator at 32°C.

### *Experiments with Monkeys*

*Experiment No. 1.—a. The Reaction of Monkey No. 1.* Monkey No. 1 used in this experiment was a young female, a cross-breed between *Macacus rhesus* and the common macacus. She was inoculated intraperitoneally on May 12th with 5 cc. of blood and 5 cc. of testicular washings (4) from a guinea pig on the third day of fever and scrotal swelling. Examination of smears from the tunica had shown few extra and intracellular organisms. Chart No. 1 illustrates the reaction of this monkey. 120 young lice had been put on her on May 8th.

Twenty-four hours after inoculation the animal's temperature rose to 105 and it was listless and refused all food. Examination revealed that she was menstruating. The next day the fever had dropped and the animal was lively again. Five days after inoculation she was shivering in her cage, was listless again and did not touch any food for nearly two days, when she slowly began to recover, improving steadily despite a continued fever and appearing healthy on the day when the fever dropped to normal. On the second day after defervescence, the lice were transferred to a normal monkey, to avoid the action of the bactericidal power of the convalescent blood in making emulsions of these lice. The lice acted normally until May 26th when large numbers began to die. Before this date only

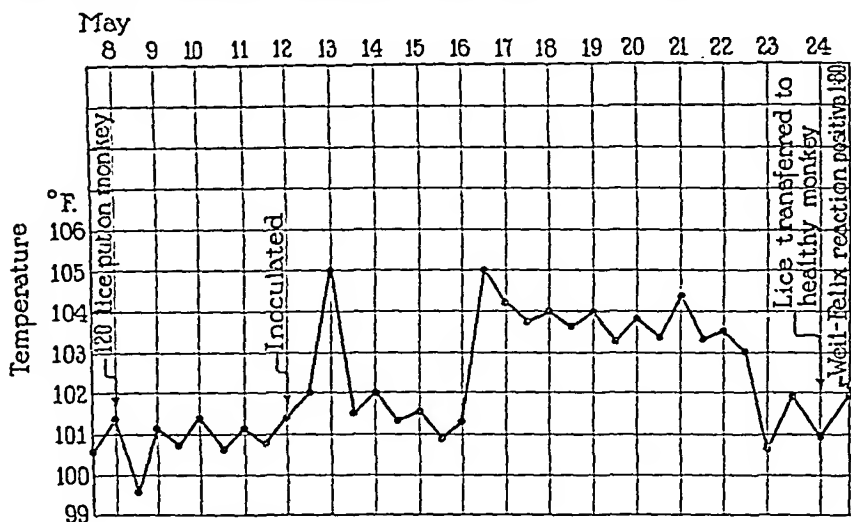


CHART 1. Monkey No. 1

four lice had died, but from then on the number of dead lice increased daily, and on May 30th, only 30 of them were still alive.

*b. The Organisms Found in Lice Fed on Monkey No. 1.* On May 24th scarce bipolar staining Rickettsiae were found in a dying louse. Every dead and dying louse was from then on examined carefully by making a smear from the dissected intestinal tract. On May 26th a second louse was found to be infected. It contained large numbers of the small red staining diplobacilliform organism described in typhus literature. On May 30th among a large number of dead lice, one was found to be very distended and discolored. It was the only one in which micro-organisms could be found. It contained very large numbers of minute red staining diplobacilli and coccoid forms and slightly larger bacillary forms exhibiting bipolar staining. Smears made from an emulsion of sixteen living lice did not reveal any organisms. Equal parts of this emulsion were inoculated into a

male guinea pig (G1) and a male white rat (Rat 1). The remaining fourteen living lice were then cut longitudinally into halves. From one side a smear was made and the other half was preserved for animal inoculation. In the smears of these fourteen lice *Rickettsia* were found in two only. One contained large numbers of the small red staining diplobacillary and coccoid forms whereas the other showed numerous pale bipolar staining bacillary forms. The halves of these fourteen lice were emulsified in a mortar, taken up with 20 cc. of normal salt solution and inoculated intraperitoneally into a male rat (Rat 2) and three male guinea pigs (G2, G3, G4).

c. *The Reaction of Guinea Pigs and White Rats Inoculated with Lice Fed upon Monkey No. 1.* Nine days after inoculation G4 showed a moderate edema of the scrotum and a temperature of 104.6 F. G2 and G3 had had irregular fever since the time of the inoculation. On the day when G4 had fever and a suspicious reaction of the scrotum, Rat 2 was killed. The examination of its organs revealed a slightly moist peritoneum and there was a fine coat of fibrinous material covering the slightly hyperemic testicles and the parietal surfaces of the tunica. Stained smears made from the surfaces of both testicles and from the tunica parietalis showed numerous heavily infected endothelial cells crowded with reddish staining minute diplobacilli and other cells containing only few slightly larger, bluish staining diplobacilli exhibiting bipolar staining. Both these forms were morphologically and tinctorially indistinguishable from the organisms seen in the lice.

With a saline solution in which both testicles of this rat had been vigorously agitated two guinea pigs were inoculated intraperitoneally. Both developed high fever and a beginning scrotal swelling sixty hours after inoculation and numerous extra and intracellular organisms were found in smears from the tunica of both animals. A separate strain of typhus (louse strain No. 1) was started from one of these animals. This strain exhibited all the characteristics of the passage strain with the exception that in the first two transfers, it showed a remarkably short incubation period and there was an unusually severe general and scrotal reaction.

G4 was killed on the third day of fever. The slight scrotal swelling had completely subsided on the second day. The autopsy was negative except for the testicles which were hyperemic and covered by a fine film of fibrin. Smears made from both sides revealed the typical cytological picture that is found in animals reacting to tabardillo but it was only after a very prolonged search that a few intracellular organisms with characteristic morphology were found within an endothelial cell.

G1, which had been inoculated with an emulsion of sixteen lice, died of pneumonia twelve days after inoculation. No indication of a typhus infection could be found at autopsy.

The slight irregular fever of G2 and G3 lasted for about ten days but there was never any sign of scrotal swelling found in spite of daily examination.

d. *Cross-Immunity Tests between the Strain Isolated from Rat No. 2 and the*

*Original Passage Strain.* There was complete cross-immunity between the louse strain No. 1 and the original passage strain. Not only did animals previously inoculated with one of them not show any fever when subsequently inoculated with the other strain, but also the scrotal reaction failed to make its appearance and no tunica organisms could be found in these animals. Guinea pigs 2 and 3, however, reacted typically after inoculation with the passage virus, and the tunica organism could easily be demonstrated in smears from the processus vaginalis. Aside from the lesions mentioned, these two animals showed evidence of a healing diffuse peritonitis.

While there can not be any doubt that the virus of typhus was recovered from a rat inoculated with an emulsion of lice infected with *Rickettsia prowazeki*, the failure of two guinea pigs to react after inoculation with an equal part of the same emulsion was difficult to explain. The circumstance that the organisms in the tunica of another animal (G4) were found only after a very prolonged search, however, was not surprising as the animal was killed on the third day of fever when occasionally extremely few or no infected cells may be found in the tunica of animals reacting to tabardillo. Two previous observations may furnish an explanation for the failure of G2 and G3 to become infected. When the present strain of tabardillo was isolated nearly two years ago (6), only one of two guinea pigs inoculated with the same amount of blood of a case of Mexican typhus reacted, whereas the other not only did not show any evidence of disease, but reacted typically when inoculated later with the passage virus. More frequent was the observation that the virus does not take easily in an animal whose peritoneal cavity is not free from another kind of infection. The reaction of the tunica constitutes a primary lesion due to the intraperitoneal inoculation which brings the virus in contact with the endothelial lining of the tunica vaginalis. Acute inflammatory processes within the peritoneal cavity seem to be decidedly unfavorable for the primary localization of the virus within the endothelial cells of the peritoneum. The failure of G1 to react is not astonishing as we had not found any *Rickettsia*-like organisms in the emulsion from the sixteen lice with which it was inoculated. Rat 1, which was inoculated simultaneously, did not agglutinate proteus X19 a week and 12 days respectively after inoculation. It was concluded therefore that none of the sixteen lice inoculated into Rat 1 and G1 contained the virus of typhus.

*Experiment No. 2.*—Monkey No. 2, a *Macacus rhesus*, was a large adult female. She was inoculated intraperitoneally on June 25th with 10 cc. of blood and nearly the entire brain of a guinea pig, killed on the fourth day of fever. At the same time an emulsion made from the scrapings of the surface of both testicles and tunicae parietales was injected, 10 cc. subcutaneously over the abdomen and 10 cc. intraperitoneally. The scrotal swelling of this guinea pig had already begun to subside when it was killed. The careful examination of six smears from both sides revealed a single endothelial cell containing the organisms. It was for this reason that we have inoculated nearly the entire brain and 10 cc. of blood in addition to the tunica emulsion.

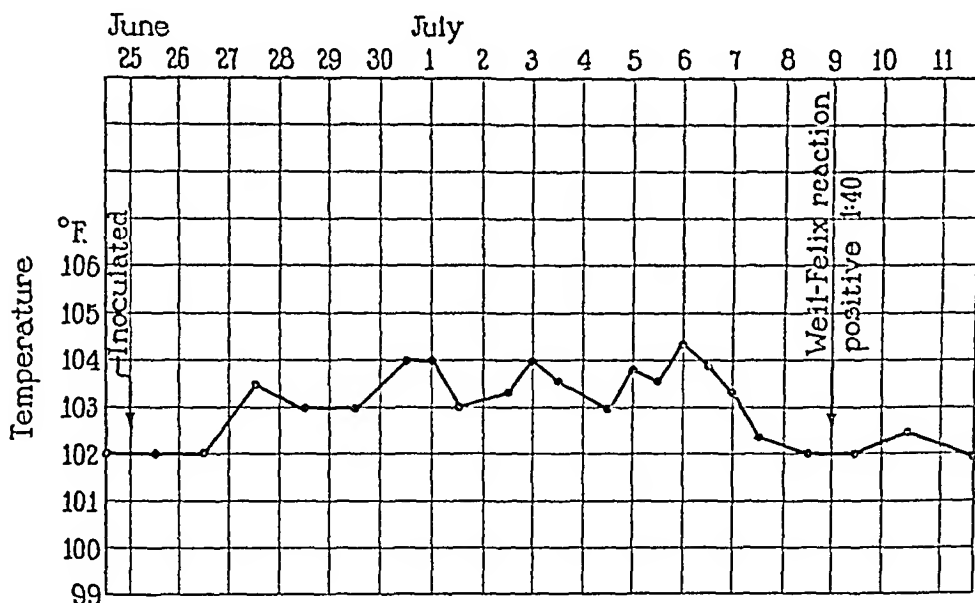


CHART 2. Monkey No. 2

Almost immediately after inoculation, this monkey showed signs of illness. She did not take any food for two days subsequently and remained crouched in a corner of her cage. On the third day, the place where the subcutaneous inoculation had been given became edematous and slightly inflamed. This reaction subsided completely within three days. The temperature of this monkey is illustrated in Chart 2.

350 lice, mostly nymphs, were put on this monkey on the day she was inoculated. They were allowed to feed twice daily as in Experiment No. 1. From the sixth day on, after they had begun to feed on this monkey, two or three of them were killed each day and examined for *Rickettsiae*. None were found. As the fever of Monkey No. 2 was very mild, we inoculated Monkey No. 3 on July 3rd and transferred the lice to the latter animal when it began to have fever. This was done on July 6th, eleven days after the lice had been put on Monkey No. 2.

This experiment was unsatisfactory and therefore not conclusive for the reason that we interrupted the louse feeding experiment too early. There is no doubt in our minds that the monkey had a mild attack of tabardillo on account of the typical local reaction (7) and a positive Weil-Felix reaction two weeks after inoculation. We transferred the lice to another monkey in order to take full advantage of the large crop of young lice carefully reared for this experiment. We were afraid that by feeding the lice unsuccessfully for a longer time on Monkey No. 2, they might have become too old to be used in another experiment, because adult lice live only about 25 to 30 days. That the lice had not become infected by feeding on this monkey and that our precaution was fully justified can be seen from the fact that *Rickettsiae* did not appear in them before the seventh day after feeding on Monkey No. 3, eighteen days after Monkey No. 2 had started to have fever.

*Experiment No. 3.—a. The Reaction of Monkey No. 3.* Monkey No. 3, a young male, was a cross-breed between *Macacus rhesus* and the common macacus. He was inoculated with an emulsion from both tunicas and scrapings from the surface of both testicles. This material was taken from a guinea pig on the third day of a very severe febrile reaction and on the second day of a very pronounced scrotal swelling. Part of the emulsion was inoculated intraperitoneally, the rest was introduced beneath the skin of the abdomen, infiltrating a large area of subcutaneous tissue. The examination of several smears from both tunicas before inoculation had revealed exceptionally numerous extra and intracellular organisms in every field of the oil immersion lens. Two guinea pigs inoculated with brain and one with a small amount of the tunica emulsion from the same animal were used as controls. Chart No. 3 gives the reaction of Monkey No. 3. All three guinea pigs developed high fever and a scrotal swelling 60 hours after inoculation. The reaction of the guinea pig inoculated with tunica was especially severe. Immediately after inoculation this monkey was an entirely changed animal. A lively and very friendly pet before, he acted very sick from the start, refusing all food for three entire days, sitting with a painful expression on his face in a corner of his cage, never budging an inch. On handling, he acted as if he had severe pain in his abdomen where the inoculation had been performed. 48 hours after inoculation an extensive inflammatory edema made its appearance at the place of injection. The central part of it became diffusely hemorrhagic the next day. This local reaction lasted for four days. Simultaneously with the swelling the fever started in this monkey. On the second day of fever, the lice were transferred from Monkey No. 2 to this monkey. They were fed upon him twice daily until the temperature had dropped to normal, when they were transferred to a

fresh monkey. Care was taken to feed the lice on the place of the local reaction as experiments with guinea pigs had shown that multiplication of the virus takes place in the skin after subcutaneous inoculation of material from the tunica. Monkey No. 3 had the severest reaction of all three animals. When the fever rose to 106 on July 9th a pronounced injection of the conjunctivae especially over the sclerae appeared. It exhibited a remarkable resemblance in respect to color and general appearance to the conjunctival reaction of human patients suffering from typhus. It persisted until the day on which the fever began to subside. At that time the monkey began to improve rapidly, returning to its normal condition within a few days.

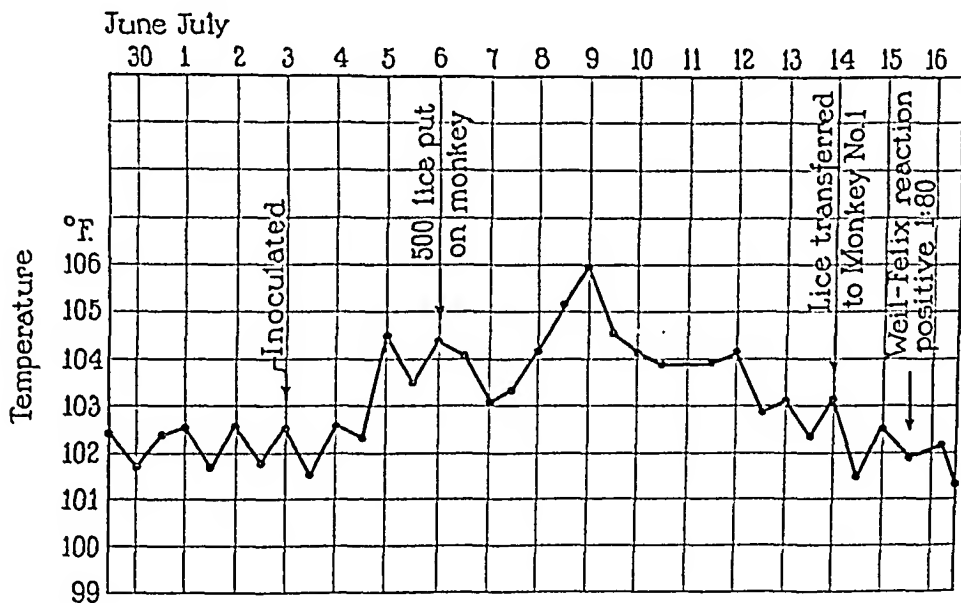


CHART 3. Monkey No. 3

*b. The Organisms Appearing in Lice Fed on Monkey No. 3.* The lice continued to feed eagerly on this monkey during the entire course of its fever with only an insignificant mortality of three or four lice daily. All lice which were found dead on opening the box were examined microscopically and also those which looked bad and refused to feed, one or two daily. No organisms were found until the seventh day after feeding on Monkey No. 3 when numerous small bipolar staining bacilli were found in teased preparations from the gut of a dying louse and from the guts of two dead lice. It was not until the tenth day that large numbers of the small red staining diplobacillary and minute coccoid forms made their appearance. The lice began to die now in great numbers and these small typical Rickettsiae were found in nearly all lice examined.

Three days after the lice had been transferred to a normal animal or twelve days after they had been put on Monkey No. 3, the box contained only 120 lice.

Sixteen were dead, 44 were dying and sixty fed normally, although the majority of them was distended and somewhat discolored. Smears made from ten lice taken at random on that day showed large numbers of the small red staining Rickettsiae in eight of them. A smear made from a greatly distended and discolored louse showed enormous numbers of these small micro-organisms.

c. *The Reaction of Animals Inoculated with Lice Fed on Monkey No. 3.* Fourteen rats and four guinea pigs were inoculated with emulsions of lice fed on Monkey No. 3. The lice were put into 95 per cent alcohol for five minutes. They were then transferred to a sterile Petri dish until thoroughly dry before making the emulsion.

The inoculations were made as follows:

July 13, 7th day after feeding: 7 lice into 2 rats (Rat 1 and Rat 2).

July 14, 16 lice into 3 rats (Rats 3, 4 and 5).

July 15, 25 " " 2 " ( " 6 and 7).

July 16, 16 " " 2 " ( " 8 and 9).

July 17, 14 " " 2 " ( " 10 and 11) and 2 guinea pigs (G1 and G2).

July 18, 60 " " 2 " ( " 12 and 13) and 2 " " (G3 and G4), and 30 lice separately into 1 rat (Rat 14).

The remaining twenty lice were fixed in Regaud's fluid for histological examination. Prior to every inoculation, a smear was made from each emulsion in order to look for Rickettsiae. This smear was made before the emulsion was taken up in saline solution. Rickettsiae were found in increasing numbers from July 13th to July 18th.

All four guinea pigs (G1, G2, G3, G4) showed fever 60 hours after inoculation and on the end of the third day a pronounced scrotal swelling developed in three of them (Figure 1) whereas the third had only a moderate swelling which increased and decreased slightly during the following three days. One animal (G3) was killed on the third day of fever. The autopsy was negative except for the pronounced and typical lesions of testicles and tunica vaginalis. The intracellular organisms were easily demonstrated in smears from both sides. From this animal a separate strain was started, louse strain No. 2. It induced the typical symptoms of tabardillo in guinea pigs and rats.

Seven rats (1, 3, 6, 8, 10, 12, 14) were killed between the 5th and 6th day after their respective inoculations. The organisms were easily demonstrated in the exudate around the testicles in all of them. In two rats (4, 11) killed after the 7th day, no organisms could be found. Although the lesions of the tunica in these three animals looked typical, the examination of smears indicated that the animals had been killed too late for the demonstration of Rickettsiae because the polynuclear leucocytes which accompany the Rickettsiae in the tunica exudate had already been replaced nearly completely by lymphocytes and large mononuclear leucocytes. From one rat which was positive a separate strain of tabardillo was started (louse strain No. 3). It produced the typical scrotal lesion in all guinea



pigs inoculated and smears from their tunicas revealed with regularity the small intracellular organisms.

The remaining five rats (2, 5, 7, 9, 13) were killed by bleeding twelve days after their respective inoculations. A Weil-Felix reaction performed with their blood gave the following results:

Rat 2 :	Positive	in a dilution of	1:20.
Rat 5 :	" " "	" "	1:20.
Rat 7 :	" " "	" "	1:10.
Rat 9 :	" " "	" "	1:80.
Rat 13:	" " "	" "	1:100.

*d. Cross-Immunity Tests between the Two Lice Strains (1 and 2), the Original Passage Strain and a Strain of Endemic Typhus from North Carolina (Maxcy Strain).* Several animals which had recovered from a typical reaction to these lice strains failed to react when subsequently inoculated with a heavy dose of passage virus and virus of the Maxcy strain respectively. Animals recovered after inoculation with the original passage strain and with Maxcy's strain proved to be immune to a subsequent inoculation with one of the louse strains. No organisms could be found in the tunica of these reinoculated animals.

The result of this experiment demonstrates more clearly than Experiment No. 1 that the virus of tabardillo which is characterized by the lesions of scrotum and tunica in guinea pigs is able to multiply in the body louse. Moreover, it demonstrates that the small intracellular organism constantly present in the tunica vaginalis of our passage strain can be recovered easily from rats and guinea pigs after the virus has passed through the louse. An observation made during these lice experiments has a special bearing on the significance of the tunica germ. Monkey No. 3 which was inoculated with material containing exceptionally numerous tunica germs had a very severe reaction and nearly all lice which were allowed to feed upon him became infected with *Rickettsia prowazeki*. Monkey No. 1 which had received blood and washings from a tunica which had shown relatively few organisms had a mild reaction and only a small percentage of lice became infected with *Rickettsia prowazeki*. Inoculation into guinea pigs and rats revealed that few of these lice contained the virus of typhus. In the tunica which was used to inoculate Monkey No. 2 only very scarce organisms could be demonstrated after a prolonged search. The reaction of this monkey was so mild that only the positive Weil-Felix reaction gave us the security that his mild fever

was really due to typhus. Not a single louse became infected with *Rickettsia prowazeki* by feeding on this monkey.

*The Inoculation of Lice by the Method of Weigl (8)*

5 cc. of human blood were drawn from a vein, defibrinated and some sodium citrate added. The testicles of a guinea pig killed within 24 hours after the appearance of the scrotal swelling were vigorously agitated in the blood. The sodium citrate was added in order to prevent coagulation of the fibrinogen which collects around the testicles during the early scrotal reaction. This mixture was inoculated rectally into 40 lice with a capillary glass pipette attached to a rubber bulb. After inoculation the lice were worn continuously by an immune person. Every day two lice were removed from the boxes for microscopical examination. The first *Rickettsiae* were found in a louse four days after inoculation. They were now found in increasing numbers in every louse examined. After the seventh day their number was enormous and the lice began to die. On the tenth day all lice which still remained in the box were found to be dying. Examination revealed that the epithelial lining of their guts was completely destroyed by *Rickettsia prowazeki*. This experiment was repeated twice with the same results. Several guinea pigs inoculated intraperitoneally with emulsions from these lice developed the typical symptoms of Mexican typhus and the tunica organism was easily demonstrated in all of them. The peritesticular exudate of three rats killed five days after the inoculation with such a louse emulsion contained enormous numbers of the small intracellular organisms.

If we compare the results of the preceding experiment with those of the feeding experiments we see that *Rickettsia prowazeki* appeared four days after a rectal inoculation and in 100 per cent of the lice, whereas in lice fed upon infected monkeys *Rickettsia prowazeki* did not appear until the seventh day and only a certain percentage of the lice became infected. This, however, is not astonishing as the organisms are always easily demonstrated during the early scrotal involvement whereas they never are found in the blood of animals reacting to typhus. More significant is the fact that the exudate around the testicles during the early scrotal reaction invariably contains several thousand infective doses of the virus of typhus. As a rule, the same amount of a tunica emulsion is at least five hundred to a thousand times more infectious than the same amount of blood. The experiments demonstrate therefore that lice inoculated rectally with concentrated virus of typhus became earlier and absolutely regularly infected with *Rickettsia prowazeki* as compared with lice which had fed on the much less infectious blood.

The following two experiments were carried out with the aim of separating the causative agent of typhus from *Rickettsia prowazeki* and from the organism in the tunica.

*The Influence of Dilution of Tunica Emulsion on the Occurrence of Rickettsia and the Causative Agent of Typhus in Inoculated Lice*

A guinea pig was killed at the beginning of the scrotal swelling and both testicles vigorously shaken in 10 cc. of citrate solution. This suspension was arbitrarily called dilution 1:10 although the amount of exudate in the scrotal sacs was far less than 1 cc. Dilutions were then made from this suspension and defibrinated human blood added to each dilution. Three separate groups containing 20 lice each were inoculated rectally, one group with a dilution of 1 to 100, one with a dilution of 1 to 1000, and one with a dilution of 1 to 10,000.

The following results were obtained:

In the first group of lice *Rickettsia prowazeki* appeared on the seventh day and the lice began to die on the twelfth day after inoculation. In the second and third groups *Rickettsia prowazeki* appeared on the ninth and twelfth day respectively. The lice of the second group began to die on the thirteenth day and several lice of the third group died on the fifteenth day. Examination of these dead and dying lice revealed large numbers of *Rickettsia* and the causative agent of typhus was demonstrated in several lice of each group separately by inoculation into guinea pigs. The tunica organism was easily found in these animals.

Sixteen days after inoculation all lice of Groups 1 and 2 had died. Of Group 3, however, eight lice continued to feed. They were killed on the twentieth day by emulsifying them in a few drops of normal saline solution. A loopful was used for making a smear. The rest was taken up with 10 cc. of normal saline solution and inoculated into two guinea pigs. No *Rickettsiae* could be found in the smear and the guinea pigs remained normal and did not show any immunity when later inoculated with an emulsion of tunica from the passage strain.

This experiment failed to separate *Rickettsia prowazeki* from the causative agent of typhus. Whereas the dilutions of 1:100 and 1:1000 of the tunica exudate infected all lice with *Rickettsia prowazeki* this organism appeared only in a small majority of the lice inoculated with a dilution of 1:10,000. Lice in which *Rickettsia prowazeki* was demonstrated harbored also the causative agent of typhus whereas eight lice of the third group which were free from *Rickettsiae* did not contain the causative agent of typhus. The amount inoculated into each louse was about 2 mgrs. Since the dilution inoculated into lice of group three was 1:10,000, the amount of tunica exudate received by each of these lice was one five millionth of tunica exudate. If Rickett-

sia prowazeki and the causative agent of typhus were two different organisms, we should expect that the inoculation of such a small quantity of tunica which is just beyond the limit where each dose contains the causative agent or Rickettsia prowazeki should separate them by the help of louse inoculation, given the extremely high susceptibility of the louse for both of them. One could of course, object that not all lice of Group 3 were equally susceptible to Rickettsia prowazeki and that our experiment does not prove that the quantity of tunica exudate inoculated into the eight lice which remained free from organisms did not contain the tunica organism. We are fully aware of this possibility. With such an assumption, however, we would presume that the degree of susceptibility of the lice for two different organisms is exactly alike, and that these two organisms always occur in the same number in an infectious material, and that the presence of one conditions the presence of the other and vice versa.

*The Incubation Period of Mexican Typhus and the Time of the Appearance of the Organism in the Tunica Vaginalis*

It is commonly thought that a febrile reaction in guinea pigs appearing earlier than six to seven days after inoculation is not due to the virus of typhus. The inoculation of concentrated tunica emulsion prepared from an animal during the early scrotal reaction is followed by fever and swelling of the scrotum within less than seventy-two hours. The Rickettsia-like organisms can regularly be demonstrated in smears from the tunica sixty hours after inoculation and repeatedly we were able to find them already at the end of the second day. We made, therefore, three successive transfers with tunica emulsions each sixty hours after the respective inoculation and injected twenty lice with an emulsion made from the tunica of an animal of the third transfer. This animal was killed fifty hours after it had been inoculated.

Result: All lice became heavily infected with Rickettsia prowazeki within eight days and the virus of typhus was demonstrated in six of them separately by inoculation into guinea pigs. In all six animals the tunica organism was demonstrated.

This experiment demonstrates that the virus of typhus and Rickettsia prowazeki make their appearance simultaneously in the tissue of guinea pigs. The experiment constitutes another fruitless attempt to separate Rickettsia prowazeki from the virus of typhus.

*The Organisms in the Lice*

The organisms found in our lice have already been referred to in our experiments as *Rickettsiae* for the sake of brevity and because they corresponded morphologically and tinctorially to the germs described in lice fed upon typhus patients by da Rocha Lima. We wish, however, to emphasize the morphological identity of the organism found in our lice with those found in the tunica of animals inoculated with the passage strain and with the three strains started from lice.

The organisms found in lice killed early after feeding on an infected monkey or after an infective enema were small pale bluish or purplish staining bacilli (with Giemsa solution) which exhibited more or less decided polar staining. As the infection of the lice progressed the minute red or purplish staining diplobacillary and coccoid forms began to show up in increasing numbers whereas the slightly larger bacilliform organisms became scarcer. Exactly the same phenomenon can always be seen in smears from the tunica of rats and guinea pigs provided that these animals are killed at the proper time. In endothelial cells which contain few organisms the larger pale bluish staining bacilli as a rule are present, whereas in heavily infected cells which are distended and about to rupture, the small red staining diplobacillary and coccoid forms are invariably met with. It seems, therefore, that the minute forms liberated from heavily infected cells by disintegration of the latter assume the form of somewhat larger bacilli after they invade a fresh cell of the louse or of an infected mammal. Wolbach, Todd and Palfrey (9) had already described this behavior in lice and our observation in guinea pigs and rats corroborates their findings in this respect. When the larger bacilliform organisms are distributed more or less evenly over the protoplasm of an infected tunica cell, or of an infected epithelial cell of the louse, they may be present in considerable numbers without the admixture of the minute forms. Very heavily infected cells, the protoplasm of which is completely filled with closely packed organisms, contain few or no large forms as a rule. Roundish or irregularly shaped colonies of closely packed organisms within large endothelial cells are also composed almost invariably of the minute red staining diplobacillary forms whereas loosely packed colonies are composed as a rule of the larger bipolar staining organism. Especially after careful differentiation of the smears with 95 per cent alcohol the bipolar staining can be seen very clearly. At the first glance one would think that the small diplobacillary forms may not be properly recognized as such on account of their great number in which individual organisms can frequently be recognized only with difficulty. This, however, can easily be ruled out by the observation of large numbers of the small forms when found strewn about cells which had ruptured while making the smears or which had already disintegrated spontaneously in situ before the smear was made. It seems, therefore, that the larger bacillary forms develop only as long as there is enough room and perhaps

ood within the cell, whereas the minute forms appear when these conditions become less favorable. We got the impression that the larger bipolar form represents the actively multiplying stage of the organism, whereas the small coccoid forms represent a resting stage. The size of a bipolar staining rod corresponds closely to the size of a diploform composed of the two minute coccoid organisms. A transitional form exhibiting hour-glass shape can frequently be observed. Arkwright and Bacot (10) and also Wolbach, Todd and Palfrey (9) described long bacillary and filamentous forms which showed indications of the formation of the small forms within their body. Similar pictures could occasionally be seen in the tunica cells of our guinea pigs and rats although much shorter ones than those mentioned by these investigators. The very large coiled forms described by other investigators in the gut of lice have never been seen by us in the tunica nor did we find them in sections or smears of lice.

#### GENERAL DISCUSSION

The result of these experiments is clear cut. Body lice fed upon monkeys inoculated with a strain of Mexican typhus carried along in guinea pigs for nearly two years became infected with the organism known as *Rickettsia prowazeki*. The inoculation of such lice into guinea pigs and rats induced in the latter animals the typical symptoms of tabardillo. The small intracellular organism found first by Mooser (1, 2) in guinea pigs inoculated with Mexican typhus and later by Pinkerton (5) in a strain of typhus from Europe and by Maxcy (3, 4) in guinea pigs and rats inoculated with endemic typhus from North Carolina could easily be demonstrated in animals inoculated with an emulsion of lice fed upon monkeys inoculated with Mexican typhus. The same results were regularly obtained with lice inoculated with an emulsion of tunica vaginalis by the method of Weigl (8).

Whereas up to the time of the conclusion of these experiments, we were only able to state that the organism found in the tunica vaginalis was constantly associated with our strain of Mexican typhus and that in its morphology and tinctorial behavior it is indistinguishable from *Rickettsia prowazeki* in lice, we know now that it accompanies the causative agent of typhus from the passage strain via monkey through the louse back into the tunica of rats and guinea pigs where it multiplies again exclusively within cells as it does in the louse, in other words, that the intracellular organism found in the tunica vaginalis of our animals is identical with *Rickettsia prowazeki*. The present experiments do not advance any startling new knowledge concerning

the etiology and pathology of typhus. They show again, however, and more impressively than had been demonstrated before that the causative agent of typhus cannot be separated from *Rickettsia prowazeki*. The causative agent of typhus seems to exist in the louse and in the mammal in one form only, namely, in the form of *Rickettsia prowazeki*. Even this is not an entirely new finding. Kuczynski (11) and especially Wolbach, Todd and Palfrey (9) had already advanced considerable evidence that the causative agent of typhus is present in man and in animals in the form of *Rickettsia prowazeki*. That their conclusions have not been universally accepted is due to the uncertainty of their method of demonstrating *Rickettsia prowazeki* in sections of mammalian tissue. This can clearly be seen from the circumstance that Wolbach, Todd and Palfrey (8) consider globular massing within endothelial cells as the most characteristic behavior of *Rickettsia prowazeki* in mammalian tissue and form their finding of *Rickettsia prowazeki* within the endothelial leucocytes which constitute the nodular lesion of typhus. Such globular massing can also be seen in smears from the tunica, but it represents only a transitional stage of the intracellular multiplication. The most characteristic finding in mammalian tissue is the occupation of the whole protoplasm of an infected cell by enormous numbers of closely packed organisms which cause great distention of the invaded cell and finally lead to its disintegration with liberation of the intracellular germs, exactly as is seen in the case of *Rickettsia prowazeki* in the louse. The same course of events can also be demonstrated within Regaud fixed tissue of guinea pigs after staining with Giemsa solution.

As long as the organisms are multiplying within an infected cell the surrounding tissue remains quiescent (12). As soon, however, as the organisms are liberated by disintegration of an infected cell, a sudden acute inflammatory reaction flares up around the disintegrating cell. The *Rickettsiae* which are not evacuated into the blood stream or which do not gain entrance into a new endothelial cell are taken up by polynuclear leucocytes, where they are digested. The leucocytes in turn are taken up by large mononuclear leucocytes which gather concentrically around them, giving rise to the typical nodule of the typhus literature. This nodule is not an early lesion but represents a healing stage of a typhus lesion and never contains any demonstrable *Rickettsiae*.

The demonstration that *Rickettsia prowazeki* multiplies within infected endothelial cells of the mammal exactly as it does in the cells of the stomach of the louse, causing great distension and finally disintegration of the invaded cells, constitutes alone strong evidence for the specificity of this organism in typhus.

All attempts to separate the causative agent of typhus from the tunica organism or *Rickettsia prowazeki* have failed completely. The following evidences have so far accumulated in respect to the specificity of *Rickettsia prowazeki* in typhus: *Rickettsia prowazeki* is only found in lice fed upon typhus patients (da Rocha Lima (13)). Its presence in lice is constantly associated with the virus of typhus (Wolbach, Todd and Palfrey (9)). Lice inoculated rectally with any material containing the virus of typhus invariably become infected with *Rickettsia prowazeki* and simultaneously there is a pronounced multiplication of the virus of typhus (Weigl (8)). Emulsions of *Rickettsia prowazeki* made from lice are agglutinated by serums of cases of typhus (Otto (14)) and also by serums of guinea pigs reacting to typhus which never give a positive Weil-Felix reaction (Weigl (8)). Organisms morphologically simulating *Rickettsia prowazeki* were demonstrated in sections of specific lesions of typhus (Kuczynski (11), Wolbach, Todd and Palfrey (9)). Recently the same organism was regularly demonstrated in smears from the tunica vaginalis of guinea pigs reacting to a strain of Mexican typhus (Mooser (2)). Pinkerton (5) found the same organism in a strain of European typhus, and Maxcy (4) in a strain of endemic typhus from this country. At this occasion it is well to remember that the virus of typhus is held back by bacterial filters (Anderson and Goldberger (15), Ricketts and Wilder (16), Olitsky (17)). The size of the specific organism must therefore lie within the range of microscopic visibility. Since in an infected louse only *Rickettsia prowazeki* can be found and since the same holds true for the tunica vaginalis, it is almost impossible to elude the conclusion that *Rickettsia prowazeki* and the causative agent of typhus are identical. All physical and chemical influences which kill the specific agent invariably kill also *Rickettsia prowazeki*. Furthermore, we have shown that the incubation period for both is the same in guinea pigs. Since the lesions in the tunica vaginalis show all the histologic characteristics of an uncomplicated typhus infection



(2) and since these lesions regularly contain no other organisms but *Rickettsia prowazeki*, we have to conclude that the latter organism is etiologically responsible for them. If we question therefore, the specificity of *Rickettsia prowazeki* in typhus, then we have also to question the specificity of the histopathology of that disease.

#### SUMMARY

Healthy lice became infected with *Rickettsia prowazeki* after feeding on monkeys inoculated with a strain of Mexican typhus. The same result was obtained in 100 per cent of lice by rectal inoculation of an emulsion of tunica vaginalis of guinea pigs reacting to the same strain. In the tunica vaginalis of guinea pigs and rats inoculated intraperitoneally with an emulsion of lice containing *Rickettsia prowazeki* the intracellular organism constantly associated with the passage strain appeared regularly. *Rickettsia prowazeki* found in lice and the organism constantly present in the tunica of guinea pigs and rats reacting to our strain of tabardillo are indistinguishable morphologically and tinctorially and their mode of intracellular multiplication is alike in every respect. It is concluded that they are identical. This organism is constantly associated with the causative agent of Mexican typhus, both in mammals and in lice, and all of our attempts to separate them have failed.

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## EXPLANATION OF PLATE 5

FIG. 1. Scrotal swelling of a guinea pig (G4, Exp. 3) inoculated with an emulsion of lice fed on Monkey No. 3. The scrotal swelling in guinea pigs is characteristic of Mexican typhus.





FIG. 1

(Mooser and Dummer: Mexican typhus and *Rickettsia prowazeki*)



# THE RECOVERY OF VACCINE VIRUS AFTER NEUTRALIZATION WITH IMMUNE SERUM

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It has been shown by Todd (1) and by Andrewes (2) that mixtures of a virus, e.g., vaccine virus with its specific antiserum which are without effect when introduced into the skin of a rabbit, become lesion-producing if diluted with Ringer's solution. Prior to the observation of this phenomenon, it was generally considered that if a virus is placed in contact with its specific immune serum *in vitro*, either a destruction of the virus takes place or union occurs between the two with loss of infectivity—so-called neutralization of the virus. The discovery of the dilution effect has completely changed current conceptions of the phenomenon.

The nature of antigen-antibody reactions in general is still unsolved. Ehrlich (3) believed that the toxin-antitoxin reaction, resembling in certain features that of the virus-immune serum mixtures, is analogous to the interaction of a strong acid and a strong base, which is complete in one direction and only imperfectly reversible. Arrhenius and Madsen (4) are opposed to this conception and regard the toxin-antitoxin reaction as resembling that of a weak acid and a weak base, leading to an equilibrium when appreciable amounts of both reacting substances and neutral salt are present. Bordet (5) disagrees with both and looks upon the toxin-antitoxin reaction as an adsorption phenomenon in which the antitoxin is distributed equally upon all of the toxin molecules. Still less is known about the nature of the immunological reaction of virus and specific immune serum than about the toxin-antitoxin neutralization.

The purpose of the present paper is to record certain observations made upon neutral mixtures of vaccine virus and its immune serum under various conditions.<sup>1</sup>

<sup>1</sup> We wish to thank Dr. D. A. MacInnes of The Rockefeller Institute for some useful suggestions.

## EXPERIMENTAL

*Source of Virus.* The vaccine virus consisted of the Levaditi (6) strain of neurovaccine, propagated in rabbits' testicles after the method of Noguchi (7). The testicles of normal rabbits were inoculated with a saline suspension of this virus and two or three days later, when they exhibited marked orchitis, the animals were etherized, exsanguinated, and the organs removed under aseptic conditions. The testicles were weighed, minced, and ground in a mortar with a small amount of sand, to which Ringer's solution or distilled water was added to make a 10 per cent suspension. The suspension was centrifuged at a low speed for a minute and the supernatant fluid decanted. The fluid was arbitrarily designated a 1:10 dilution of the virus. The procedure of preparation of the suspension was carried out under sterile conditions.

*Immune Serum.* This was obtained from rabbits which had recovered from characteristic experimental vaccinia. The serum was procured, pooled, and bottled without preservative, using aseptic precautions. It was not inactivated and was kept at a temperature of about 4°C.

*Test Animals.* Healthy, full-grown, white rabbits were used in all tests. The hair was removed from the flanks by careful shaving or by barium sulphide, preferably by shaving. The inoculations, with few exceptions, were made intracutaneously. If care is used, circular lesions can be obtained with the endermic technique. The lesions were measured up to three days after their appearance, at which time secondary lesions generally occurred.

*Preparation of Neutral Mixtures.* Neutral mixtures of vaccine virus and its immune serum were usually prepared by adding to a given amount of antiserum an equal quantity of a 1:200 or 1:400 dilution of suspension of fresh virus. No incubation was required, since neutralization occurred instantly.

*Experimental Results**Cataphoresis Tests*

Our intention in performing cataphoresis tests on neutral virus-immune serum mixtures was to note whether the virus could be separated from its antiserum by means of an electric current. A typical experiment, which on repetition gave similar results, is shown in Protocol I.

*Protocol I.* The method of cataphoresis as applied here has already been fully described (8). As preliminary tests, we studied by means of cataphoresis vaccine-virus infected testicles of rabbits in suspensions at  $\text{pH} = 6$  to 8. The virus was uniformly recovered at the anode. The prior observations of Douglas and Smith (9) that the virus, or material containing the virus, migrates in an electrical field and carries an electronegative charge was thus confirmed.

There were prepared 100 cc. of equal parts of virus and antiserum at a pH = 7.8, which was proved ineffective by animal inoculation test. After three hours' cataphoresis at 4.8 milliamperes with a potential drop of 117 volts, active virus was obtained at the anode, as revealed by intracutaneous inoculation of anodic material in the rabbit.

In repeated experiments, the pH of the neutral mixtures of virus and immune serum measured, respectively, 6, 7.5, 7.6, and 8; the milliamperage varied from 3 to 4.5; the potential drop from 115 to 118 volts (or from 38.3 to 39.3 volts per tube) and time constantly three hours. In all these tests the virus was again revealed at the anode.

The results obtained from repeated tests show that from mixtures which are ineffective as such, active virus can be recovered from anodic material after cataphoresis. Hence it can be inferred that in neutral mixtures of the virus and its antiserum, the former is not destroyed by the serum.

#### *Influence of Electrical Charge on Union*

Bedson and Bland (10) have recently reported that they have been able to determine the electrical charge of herpes and vaccinia virus particles by measuring the ascent of the particles into negatively charged blotting paper dipped into suspensions of the viruses for given periods of time. It was thought that a change in the electrical charge of the vaccine virus particles might be demonstrated by this method, if union occurred with its specific antiserum.

An example of the results obtained in five separate experiments is given in Protocol II.

*Protocol II.* 15 cc. of a 1:200 dilution of fresh testicular neurovaccine virus in phosphate buffer at pH = 7.5 were prepared. To this was added 15 cc. of the serum from a rabbit recovered from neurovaccine-virus infection. The mixture was divided into two portions and in each was suspended to the depth of 0.5 cm. a strip of blotting paper 1 cm. wide and 10 cm. long. After one hour the strips were removed and cut into centimeter lengths. The paper corresponding to the third, fourth, and fifth centimeter was used for animal test. In the case of the first strip of blotting paper, the different lengths were applied directly to scarified areas on the flank of Rabbit A, and then rubbed into these areas. In the instance of the second strip of blotting paper, the different lengths were ground with 3 cc. of Ringer's solution, and 0.5 cc. of the supernatant fluid was injected intracutaneously into the flank of Rabbit B. Rabbit A was unaffected, but Rabbit B developed characteristic vaccinal lesions at the sites of inoculation. For controls in each experiment, virus by itself and virus plus normal rabbit serum were used.



From these tests, which, on repetition, gave similar results, it follows that at a hydrogen ion concentration of 7.5, the neutralization of vaccine virus by its specific antiserum does not result in an appreciable change in the electrical charge carried by the virus particles.

### *Leucocytic Test for Union*

Recently Smith (11) has shown that vaccine virus, injected into the circulation of a completely immune rabbit, disappears rapidly, and that the removal from the circulation is a consequence of the fixation of the virus by leucocytes. This test was repeated in a rabbit immune to vaccinal infection, as revealed by its resistance to dermal and corneal inoculation of the virus.

5 cc. of a 1:50 saline suspension dilution of virus were injected into the margin a ear vein. Three hours later the animal was bled from the heart<sup>2</sup> to the extent of 40 cc. The blood was mixed with 4 cc. of 20 per cent sodium citrate to prevent clotting and after centrifugation, the white cells were collected from the buffy layer, washed several times in warm physiological saline solution, and a thick suspension injected endermically into a rabbit. The whole blood, washed red cells, plasma, diluted whole blood, and diluted plasma were used as controls. The latter produced no lesions but the white blood cells induced within forty-eight hours at the site of inoculation a characteristic vaccinal lesion.

Thus this test confirmed Smith's observation in indicating that in an immune animal the virus is removed from the circulation by the leucocytes and, therefore, is not found to be inactivated in the presence of immune plasma.

### *Effect of Storage in the Cold on Union*

It was observed that now and then certain ineffective mixtures of virus and its immune serum, kept at a temperature of about 4°C. for periods of from one to eight weeks, again became potent. In one of five such occurrences, among twenty-seven neutral virus-immune serum mixtures, activity was revealed in a dilution of 1:1 million, even though the mixture had been kept in the icebox for four weeks. The original titration of the virus showed potency in a dilution of 1:10 million.

Although the results are not constant, their frequency of occurrence attracts attention to the fact that the union between virus and its antiserum, if it exists at all, is a loose one.

<sup>2</sup> All operative procedures on animals were made with the aid of ether anesthesia.

*Dilution Tests*

A series of twenty-seven tests was made to determine the effect of dilution upon neutral mixtures of vaccine virus and immune serum. The results can be summarized as follows:

*Protocol III. Methods.* Serial dilutions of the mixtures were prepared ranging from 1:20 upwards but, in most instances not carried beyond 1:160, since it

TABLE 1  
*Size of Lesions on First, Second, and Third Days*

Amount injected (cc.)	Dilution of immune-serum			
	1:20	1:40	1:80	1:160
	Size of lesion (mm.)			
First day				
0.05	3	0	6	10
0.1	4	6	8	12
0.2	6	8	9.5	13
0.4	6	8	12	21
Second day				
0.05	6	6	11	15
0.1	8	12	13	17
0.2	7	14	15	17
0.4	12	16	18	22
Third day				
0.05	6	6	10	14
0.1	9	12	14	14
0.2	7	14	16	22
0.4	14	16	20	28

was found that beyond this dilution no visible evidence was apparent of any influence of the serum upon the size of the lesion. The diluents used were physiological salt solution, Ringer's solution, Locke's solution, sterile broth, normal serum, whole blood, and distilled water. The range of pH extended from 5.5 to 8.4.

As a rule, 0.5 cc. of each dilution was injected along a line in the shaved flank of a rabbit. A titration of the virus was always made on the opposite flank. With precision in the technique, the endermic inoculation yields almost always circular lesions.

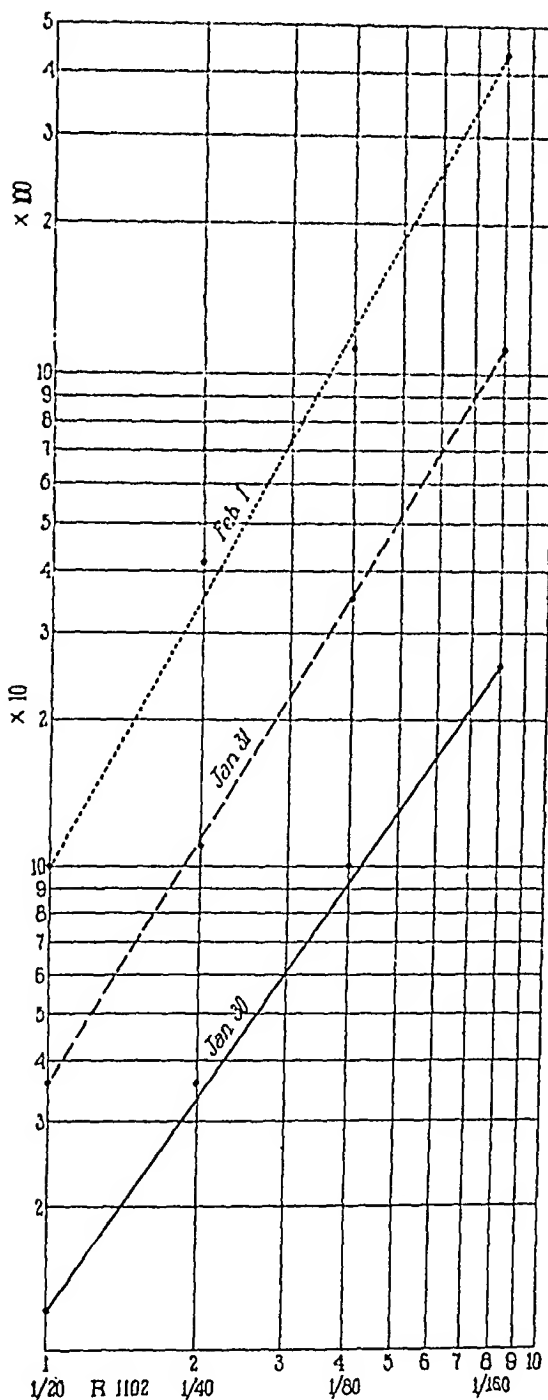


CHART I. Showing the quantitative relations of dilution and size of lesions in logarithmic curves.

It soon became apparent from the varying size of the lesions that, up to a dilution of 1:160, a definite relation existed between the diameter of the lesions and the dilution of the immune serum, since vesicles of the same size could usually be obtained by diluting neutral mixtures beyond 1:160, at which point the immune serum ceased to exert any influence upon the size of the lesion. A typical example is given in Experiment A.

*Experiment A.* Dilutions of neutral mixtures of vaccine virus and its antiserum were prepared in such a manner that while the concentration of the serum varied in the different dilutions, the concentration of the virus remained constant. 0.05, 0.1, 0.2, and 0.4 cc. of each dilution was injected into the shaved flank of Rabbit A and with the appearance of the lesions measurements of their diameters were made until the secondary eruption appeared. These measurements are given in Table 1.

In Chart I is given the remarkable quantitative relations between dilutions of a mixture of virus and its antiserum and the size of the lesions, in the case of another rabbit. The abscissae are proportional to the dilution of the mixture; the ordinates are proportional to the areas of the lesions.

It was found that if total amounts of diluted mixtures were inoculated, the lesions varied in size in proportion to the dilution of the antiserum, the virus being kept constant in amount at each injection by diluting 0.01 cc. of a neutral mixture with 0.09 cc., 0.19 cc., 0.39 cc., and 0.79 cc. of distilled water respectively. As a control, 0.01 cc. of virus suspension was diluted with an equal amount of distilled water. The total volumes were then inoculated into the flanks of rabbits.

*Over-Neutralized Mixtures.* When over-neutralized mixtures comprising one part of virus plus 2, 3, or 4 parts of immune serum, which was still in the range of effectiveness, were diluted with appropriate amounts of distilled water, the same dilution effect was observed. However, with these mixtures the dilution had to be increased in proportion to the degree of over-neutralization before a characteristic result could be obtained. For example, 1:80 of a mixture over-neutralized four times produced a certain sized lesion, but 1:20 of a mixture exactly neutralized, and in which the same materials were used, yielded the same sized lesion.

It would appear from these experiments that the size of the lesion bears a direct relationship to the dilution employed. Furthermore, it is possible to estimate the probable size of a lesion which may be induced by diluted mixtures of virus and its antiserum, if given the size produced by a known dilution.

#### DISCUSSION

Early in the course of the experimental work presented here, it was thought that the effects produced by dilution of mixtures of vaccine

virus and its specific antiserum probably follow the law of mass action (12). Later, with more experimental data at hand, it became apparent that while the observed phenomena might be interpreted as following this law, no conclusive evidence could be noted of union occurring between the virus and its immune serum. This might well, however, be due to very weak combination between the two components, the compound being at all dilutions in equilibrium with varying amounts of its constituents.

The results of cataphoresis experiments demonstrate that the virus can migrate in a neutral, ineffective mixture of virus and its antiserum in a similar manner as in a saline or aqueous suspension. Furthermore, when the virus particles are in contact with antiserum, there appears to be no change in its electrical charge. In addition, if virus is introduced into the circulation of an immune rabbit, it is apparently taken up by the leucocytes and is not found to be in combination with the blood serum. While this method of protection of the animal, first observed by Smith, argues against a conception of stable union between the virus and immune serum, it cannot be, by itself, regarded as conclusive evidence against a loose combination with an equilibrium such as is described above.

The tests show definitely that the dilution of a neutral mixture of vaccine virus and immune serum renders it lesion-producing and that the size of the lesions bears a direct relationship to the amount of diluent employed. The readiest explanation of this striking effect is that given above, that there is a loose combination between the virus and serum, the separation of which follows relations which can be deduced from the law of mass action.

#### CONCLUSIONS

1. When vaccine virus and its specific antiserum are brought together, no evidence of stable union between them can be determined by the experimental methods employed.

2. A definite relationship exists between the degree of dilution of a neutral vaccine virus-antiserum mixture and the size of the lesion produced by endermic inoculation of rabbits of any given dilution.

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# THE EPIDEMIOLOGY OF FOWL CHOLERA

## EXPERIMENTAL STUDIES

### I. INTRODUCTION

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The following studies on experimental epidemiology carried out during the past few years have been concerned chiefly with the disease fowl cholera. A report of this work is presented in detail in the succeeding papers (1 a-d); in this introduction, a brief statement will be made of the purpose and scope of the work, the methods employed, and results and conclusions obtained.

The investigation was undertaken primarily to determine whether bacterial infections in general spread according to one and the same type of mechanism. It had been found that in communities of mice or rabbits in which certain intestinal and respiratory infections prevail, epidemics occur in response to a characteristic sequence of events, disturbing the balance between the resistance of the community and the quantity of available microbes (1 e). The steps in the evolution of such epidemics appear to be as follows: first, some influence can be demonstrated in these communities which operates to decrease the resistance factor. A *fall in resistance* causes a reciprocal rise in the number of carrier individuals and in the amount of infectious material available. This *increase in the dosage* factor is followed by the epidemic outbreak. No significant enhancement in the virulence of the microbes is demonstrable. So consistent are these findings that their further operation was tested by similar studies of another animal disease.

Fowl cholera was chosen for study because it is regarded as a classical example of an epidemic disease. Its characteristics are well-recognized.



The disease occurs, according to Manninger (2), wherever poultry are raised in any great number. It is said to assume two forms: one, explosive, acute, and highly fatal, in communities hitherto unexposed; the other, a mild, endemic form attended by occasional, rather severe outbreaks. *P. avicida* is believed to exist both in a saprophytic form of low virulence and as a highly virulent parasite in the tissues of an occasional "carrier" fowl (2). The portal of entry is believed by field observers to be the gastrointestinal tract, although attempts to induce the disease experimentally by feeding have commonly failed (3). The lesions found in fowls dying of the acute form of disease are not extensive. They consist of sero-fibrinous exudate in the pericardial sac, subserous petechial hemorrhages in the pericardium and intestine, and congestion and sero-fibrinous exudate in the lungs. When the affected fowl survives longer, one finds fibrinous pneumonia and necrotic foci in the liver. In the chronic disease, caseous pneumonia, pulmonary abscesses, periartritic, subcutaneous, and intramuscular abscesses are encountered. Moreover, localized lesions, such as edema of the wattle and some forms of nasal, sinus, and ocular roup have been considered manifestations of fowl cholera (4). *P. avicida* is known to possess definite characteristics common to the Pasteurella group. But the serological relationships of cultures from different sources and of Pasteurella organisms of other animals are poorly understood.

These facts summarize what is known of the epidemiology of fowl cholera. The present studies were designed to gain a more complete understanding of its mode of spread. For this undertaking, three general methods of investigation were employed: A) bacteriological and pathological tests; B) field observations, and C) studies of epidemics experimentally induced in poultry flocks. Each was applied to a definite phase of the general problem. The bacteriological and pathological tests were made to determine 1) the characteristics of strains of Pasteurella obtained from typical fresh cases of the disease, 2) the relation of these strains to the so-called "surface," "saprophytic" forms of the organism, 3) the portal of entry into the fowl, and 4) the varieties of host response to these microbes. A field study of the spontaneous disease in poultry flocks was undertaken to ascertain 1) the amount and character of the infection, 2) monthly fluctuations in its prevalence, 3) relation of *P. avicida* nasal carrier rate to spread of the disease, 4) the significance of different strains of *P. avicida*, and 5) differences in response of individual birds to the infection. An experimental study of fowl cholera epidemics is being made under controlled conditions whereby communities of healthy fowl are established and strains of *P. avicida* introduced, a technique permitting

direct determination and measurement of the factors concerned in the rise and fall of epidemic outbreaks.

The results of A) the special studies of *P. avicida* are presented in the following paper by T. P. Hughes (1 a); a preliminary report has already been issued (1 f). The findings are as follows: 209 strains of *P. avicida* from fresh cases of fowl cholera arising on widely separated poultry farms proved to be closely identical in cultural and serological behavior. On the basis of colony formation, however, three groupings were made: "fluorescent," "blue," and "intermediate" types. The "fluorescent" form, stable in suspension, with low, narrow zone of agglutination in acid buffers (pH 2.4-3.0), was found only in association with severe epidemics, and was relatively highly virulent and non-vegetative. The "blue" colony type, unstable in suspension, with wide zone of acid agglutination (pH 2.4-5.0), was found to be commonly associated with endemic cholera and was of relatively low virulence and high vegetative capacity. The "blue" colony type was obtained also *in vitro* from cultures of "fluorescent" type organisms grown under unfavorable conditions. The third colony type, designated "intermediate," proved somewhat stable in suspension, usually with a wide acid agglutination zone, and was "intermediate" in virulence and vegetative capacity. This variety was found in communities in which severe epidemics existed. Apparently the fluorescent colony form can be transformed into a blue colony type which may or may not be identical with the "blue" colony types found in communities where fowl cholera is endemic. Attempts to change "blue" colony types to "fluorescent" have been unsuccessful. The limit of variability of the "intermediate" colony type has not been determined. Under some conditions it resembles the "fluorescent" form; under others, the "blue," and yet attempts to effect a permanent transformation have not succeeded. The serological uniformity of *P. avicida* indicated by these studies may be apparent rather than real. Possibly, specific substances, undetected and not measurable, are present.

The results of special studies on portal of entry of *P. avicida* and host reaction to these organisms are presented in paper III by T. P. Hughes and I. W. Prichett (1 b), and in the preliminary report (1 f). They point out that fowl cholera is to be regarded as a respiratory infection which manifests itself in various ways by bringing about a)

a "carrier" state in the nasal passages of healthy individuals, b) an involvement of sinuses and nasal passages in others, as well as c) the typical manifestation of the acute septicemic process.

The results of B) field studies of the spontaneous disease in poultry flocks are contained in papers IV and V by I. W. Pritchett, F. R. Beaudette, and T. P. Hughes (1 c, d.) The findings differed according to whether the infection had been endemic in the flock for some time, or introduced relatively recently. The infection, when present in endemic form, varied with the season, increasing in amount and severity during the autumn months to a winter maximum, and then declining during the summer season to a minimum. Furthermore, its prevalence and tendency to spread varied with the number of nasal carriers: a decrease in carrier rate being accompanied by a decrease in disease mortality and in the occurrence of the local infections, roup, catarrh, and edema of the wattle. In these communities, the "blue" colony type of *P. avicida* was recovered. The virulence of strains from fatal septicemic cases, from local lesions of survivors, and from the nasal clefts of healthy carriers, was similar and relatively low. *P. avicida* infection in flocks of poultry with no history of previous cholera assumed the form of severe epidemics. The outbreaks arose during the winter months. The daily death rates were high; post-epidemic carriers among the survivors were uncommon. Following an epidemic, the infection tended to die out. The strains of *P. avicida* recovered were of both the "fluorescent" and "intermediate" colony types and of high virulence.

C) The observations on "experimental" epidemics of cholera are still in progress and are withheld for further consideration.

The studies so far carried out indicate that the epidemiology of fowl cholera rests upon essentially the same basis as that of rabbit pasteurellosis (1 e). In each instance, the severe epidemic form of infection is associated with a relatively virulent type of organism which survives with difficulty in the tissues of the host, whereas the endemic disease is associated with strains of relatively low virulence and high vegetative capacity. In fowl cholera, as in other animal diseases studied (1 e), the spread and severity of infection appear to be controlled by the resistance of the host and the dosage of the organisms.

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# THE EPIDEMIOLOGY OF FOWL CHOLERA

## II. BIOLOGICAL PROPERTIES OF *P. AVICIDA*

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### PLATE 6

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At the outset of the present studies of fowl cholera, attention was directed to the etiological agent, *Pasteurella avicida*.<sup>1</sup> The early investigations of this organism are chiefly of historical significance; reports of its biological characteristics, its range of variation and adaptability, and indeed, of the means for its exact identification are incomplete and contradictory.

The unsatisfactory state of this information can be emphasized by a brief summary of the literature. Following the early investigations of Perroncito (1), Toussaint (2), Pasteur (3), and Kitt (4), *P. avicida*, together with similar bacteria from other animal hosts, were placed together and designated the "Pasteurella" or "hemorrhagic septicemia" group (5). But later investigators disagreed as to the properties of these organisms. Thus, *P. avicida*, according to Schirop (6) and others (7-12), ferments dextrose, saccharose, and mannite without the production of gas. Kaupp and Dearstyne (13), on the other hand, found dextrose alone to be acidified; Tanaka (14), maltose, in addition to the above; Higgins (15), acid production on lactose litmus agar. Indol formation in peptone water was observed by all the investigators mentioned, save Kaupp and Dearstyne (13) and Lignieres (16). Theobald Smith and others (17-18) report the organism to be highly virulent for fowl and rabbits; Turner (19) regards this as a diagnostic criterion. Wide discrepancies in results of serological studies are indicated by the reports of Bushnell (20), which state that less than 50 per cent of the strains examined were capable of acting as antigens, while Fitch and Nelson (21), on the other hand, divided their strains into definite groups according to their agglutination in specific sera.

Such conflicting reports made necessary a further study of *P. avicida*. To this end, 209 strains were collected from autopsies on

<sup>1</sup> Synonyms: *Bacillus avisepticus*, *Pasteurella avium*, *Coccobacillus avicida*, *Bacillus bipolaris aviseptica*, and *Pasteurella cholerae gallinarum*.

recent cases of undoubted fowl cholera. Thirty-one of these strains came from a commercial poultry farm on Long Island (Paper IV), 172 from six scattered farms in New Jersey, 4 from poultry in Northern New York, and 2 from Kansas. One further strain, "Pa," obtained from Dr. Theobald Smith, came originally from a farm in Pennsylvania. These 210 strains were submitted to routine and special examinations, the results of which are contained in the following paragraphs.

### *General Characteristics*

*Morphology.* All strains appeared as small, rounded, bipolar bacilli, decolorizing in Gram stain and were non-motile. The bacilli seemed relatively large in blood films from infected fowls, and in films stained by Wright's method (Fig. 1). Involution forms occurred in old cultures, small, faintly colored, coccoid forms predominating. Attempts to demonstrate capsules with special stains were not successful, but on numerous occasions the highly virulent, so-called "fluorescent" colony forms showed a clear peripheral zone resembling that of an unstained capsule.

*Growth in Fluid Media.* The bacilli grew well in infusion broth plus a trace of hemolyzed rabbit or chicken blood. Four hours after loop inoculation of the fluid, turbidity was perceptible; after fifteen hours, it was maximal. Pellicle formation appeared occasionally in 3-4 day cultures, but "granulation" and sedimentation did not occur. Asparagin synthetic media did not support growth; Dunham's peptone water gave feeble and retarded growth. Growth rate was enhanced, however, when unheated fresh chicken tissue was added to infusion broth.

*Carbohydrate Fermentation and Indol Production.* The carbohydrate media was made up of Dunham's peptone solution plus 0.5 per cent sugar and 1.0 per cent Andrade's indicator. This was tubed in 5 cc. quantities, together with a small, inverted tube to detect gas production. All strains acidified dextrose, saccharose, and mannite without the formation of gas. The acidity was slight and tended to decrease as artificial cultivation was prolonged. One exception to this behavior was noted in the case of twenty strains from a single flock which showed, in addition to the above, acid in xylose. All strains

gave a positive indol test when grown 2+ days in Dunham's peptone and examined by Ehrlich's method.

*Colony Morphology.* The cultures when grown on infusion agar plus a trace of hemolyzed blood showed three distinct forms of colony. Colony morphology was found later to be associated with other definite properties and came to be used as a means of classification. Hence, considerable attention was devoted to the behavior of these strains on solid media.

The first colony type was designated "fluorescent." It is relatively large and when seen by transmitted light, is opaque and fluorescent. By reflected light, it is greyish-white and moist (Fig. 2). Three strains from the Long Island farm, those from northern New York, the Kansas, and "Pa" cultures, all "epidemic" strains, formed fluorescent colonies.

The second colony type was called "blue." It is much smaller than the fluorescent form, relatively transparent, grey-blue, and without fluorescence (Fig. 2). All strains from the New Jersey flocks in which fowl cholera was endemic were of the "blue" colony type. This form occurred also when the fluorescent type strains were grown for extended periods in artificial media or with specific antisera.

The third form of colony has been termed "intermediate," because of its partial resemblance to both the "fluorescent" and "blue" types. Under optimum conditions it may be described as similar to the "fluorescent" colony, but less opaque and less fluorescent. Colony appearance of the "intermediate" type is variable, however. When fifteen hours old, it seems typically "fluorescent;" at forty-eight hours, quite "blue." When colonies of this type are crowded, those at the periphery appear "fluorescent" at twenty-four hours, and the central ones "blue;" later, all become "blue." Frequently "ring" colonies are seen, composed of a "blue" core and "fluorescent" ring. The "intermediate" colony type strains were obtained from a single farm on Long Island during epidemic and post-epidemic periods.

The permanency of these colony types was tested on media, in sera, and in the animal body. When passed on solid media, "blue" colony forms remained "blue," "intermediates" varied as described in the preceding paragraph, but did not actually change to either of the other two types. The "fluorescent" type, on the other hand, did not



alter on blood agar, but, on infusion agar after eight passages, showed a mixture of "fluorescent" and "blue" colony forms. In fluid media the variations were similar, except that "fluorescent" types changed to "blue" more rapidly. Passage in antisera did not change the colony form of the "blue" nor "intermediate" type, but the "fluorescent" forms were replaced by "blue" colonies. Direct passage in fowls by intrathoracic injection failed to change the colony types. At present, therefore, it seems that "blue" colony types are permanent, and that "intermediate" forms as well, although varying in appearance, do not change their type. "Fluorescent" colony strains, on the other hand, growing under unfavorable circumstances, change to the "blue" colony form.

*Acid Agglutination Zone.* The behavior in acid buffer solutions of seventy strains was tested according to the method of Northrop and de Kruif (22). On centrifuging it was found that the "blue" colony type strains sedimented easily, whereas suspensions of the "fluorescent" colony strains were very stable. This difference was in accordance with the results of the acid agglutination tests (Table I) which showed a characteristic zone for each type. The "fluorescent" colony strains flocculated at pH 2.4 or not at all. The "blue" colony strains, on the contrary, showed either a wide zone, pH 2.4-5.4, or a zone well toward the alkaline side. Flocculation of the "intermediate" colony forms was limited to a more narrow region toward the acid range of the scale, pH 2.4-4.0.

*Serum Agglutination.* The antigenic relationships of the 210 strains were studied by direct and reciprocal agglutination and absorption tests.

Antisera were prepared by injecting rabbits intravenously with washed saline suspensions of organisms taken from agar plates and preserved with 0.2 per cent phenol. The injections were given on five successive days, with doses increasing regularly from 0.1 to 0.5 cc. Two additional injections of 0.5 cc. were administered at five day intervals. After a final rest period of ten days, the animals were bled.

The first test was made with a "Pa" strain antiserum. Antigen-antibody mixtures were read after a three hour period in a water-bath at 56°. All but ten strains were agglutinated. These non-agglutinating cultures were either "fluorescent" or "intermediate" colony forms with narrow acid agglutination zones.

Further tests were made with the "fluorescent" and "intermediate" colony strains to determine if possible whether they were antigenically unrelated to the other cultures or combined with specific antibody and unable to flocculate. In the first place, it was found that "blue" colony variants from the "fluorescent" forms agglutinated to titre in the "Pa" serum. Again, antisera prepared from two "fluorescent" and two "intermediate" colony forms were ineffective toward their

TABLE I  
*Acid Agglutination of P. avicida*

Colony type	Source	Range of pH	Number of strains
Fluorescent	Autopsy	No agglutination	2
"	"	2.4 only	1
Blue variant	Fluorescent	2.4-5.4	2
" "	"	2.4-4.6	1
Blue	Autopsy	2.4-5.4	18
"	"	2.4-5.0	1
"	"	3.0-5.0	22
"	"	3.0-5.4	5
Intermediate	"	2.4 only	2
"	"	2.4-3.0	1
"	"	2.4-3.2	2
"	"	2.4-3.5	1
"	"	2.4-4.2	1
"	"	3.0-4.0	3
"	"	3.0-4.2	7
"	"	3.2-3.5	1

homologous antigens, but brought about strong agglutination of the "Pa" strain. Finally, reciprocal absorption tests were made according to the method of Krumwiede (23) to determine whether the non-agglutinating strains could absorb agglutinins from heterologous sera and, conversely, whether the heterologous strains could absorb agglutinins from the sera of the non-agglutinating strains. The results of three such tests are given in Table II. Strains 631 and 638 are representative of the "fluorescent," non-agglutinating form; Strain 728 is an example of the "blue" agglutinating form; Strain

TABLE II  
*Reciprocal Absorption Tests with P. avicida*

Serum	Antigen	Result
<i>"Pa" and 728</i>		
"Pa"—unabsorbed	"Pa"	Agglutination 1:320
" " " "	728	" 1:320
" —absorbed with "Pa"	"Pa"	No agglutination.
" " " "	728	" "
" " " 728	"Pa"	" "
" " " "	728	" "
728 —unabsorbed	"Pa"	Agglutination 1:320
" " " "	728	" 1:320
" —absorbed with "Pa"	"Pa"	No agglutination.
" " " "	728	" "
" " " 728	"Pa"	" "
" " " "	728	" "
<i>"Pa" and 631</i>		
"Pa"—unabsorbed	"Pa"	Agglutination 1:320
" " " "	631	No agglutination.
" —absorbed with "Pa"	"Pa"	" "
" " " "	631	" "
" " " 631	"Pa"	" "
" " " "	631	" "
631 —unabsorbed	"Pa"	Agglutination 1:1280
" " " "	631	No agglutination.
" —absorbed with "Pa"	"Pa"	" "
" " " "	631	" "
" " " 631	"Pa"	" "
" " " "	631	" "
<i>"Pa" and 638</i>		
"Pa"—unabsorbed	"Pa"	Agglutination 1:320
" " " "	638	No agglutination.
" —absorbed with "Pa"	"Pa"	" "
" " " "	638	" "
" " " 638	"Pa"	" "
" " " "	638	" "
638 —unabsorbed	"Pa"	Agglutination 1:600
" " " "	638	No agglutination.
" —absorbed with "Pa"	"Pa"	" "
" " " "	638	" "
" " " 638	"Pa"	" "
" " " "	638	" "

"Pa" an "intermediate" agglutinating form. Strains 631 and 638 did not agglutinate in homologous or heterologous sera; however, they did absorb agglutinins from homologous and heterologous sera. Furthermore, antibodies in sera from these non-agglutinating strains were absorbed by the homologous and heterologous strains. The test with the "blue" form 728 is included as a control, showing the complete absorption of agglutinating strains.

The antigenic relationship of chicken and rabbit strains of *Pasteurellae* was tested by titrating a) rabbit cultures, Rivers D and DC 30 (29) in chicken strain antisera 638 and "Pa," and b) fifteen fowl cholera strains, representing "blue," "fluorescent," and "intermediate" colony forms in rabbit strain, Rivers D, antiserum. No agglutination occurred, indicating that the fowl cholera strains differ antigenically from the rabbit strains tested.

*Complement Fixation.* To test further the antigenic relationship of strains of *P. avicida*, the complement fixation reaction was employed. Thirty strains were examined for their complement fixing power with "Pa" serum and a lesser number with sera from non-agglutinating strains previously described.

In general, the method described by Bull (24) was followed. The organisms to be used as antigens were well washed and suspended in salt solution. Their anti-complimentary titre was determined by finding the amount of suspension that would destroy the complement in 0.2 cc. pooled guinea pig serum diluted 1:10. The mixture of complement and bacteria was incubated at 37° for one hour, then tested with anti-sheep cell hemolytic system. One-third of the anti-complimentary titre was used as the antigenic dose in the final test. This amount of suspension was mixed with 0.5 cc. of the "Pa" serum, used in dilutions from 1:16 to 1:1200. Two units of complement were added, the tubes brought to a uniform volume of 1.5 cc., allowed to incubate at 37° for one hour, then left overnight in the icebox. The hemolytic system was added and incubated for one hour at 37°. Tubes in which hemolysis was entirely inhibited were considered positive.

The results of these complement fixation tests are given in Table III and compared with corresponding agglutination titres. The titre of various strains with other sera is presented in Table IV. The in-agglutinable "fluorescent" colony strains proved fairly reactive in their ability to fix complement, and the "Pa" antigen fixed complement in various sera to the same extent as the homologous strains.

TABLE III  
*Results of Complement Fixation and Agglutination Tests*

	Number of strains	Maximum effective dilution of "Pa" serum	
		Agglutination	Complement fixation
Fluorescent form	1	0	25
	1	0	256
	1	0	40
Blue form	1	1280	160
	1	1280	320
	2	2500	320
	2	2500	160
	2	2500	512
	1	2500	640
Intermediate form	2	0	256
	1	40	20
	1	50	40
	1	80	25
	2	80	50
	1	80	75
	1	160	40
	4	160	50
	1	160	160
	1	320	50
	3	640	640

TABLE IV  
*Complement Fixation Tests with Organisms of Different Colony Forms*

Antigens	Maximum serum dilution showing complete fixation				
	Sera				
	"Pa"	770	773	629	638
"Pa"	50	75	200	300	900
770 ("Blue")	75	25	100	50	200
773 ("Blue")	50	100	200	100	200
629 ("Fluorescent")	40	50	100	100	600
638 ("Fluorescent")	20	25	75	100	600

These results are regarded as further evidence of the antigenic similarity of strains of *P. avicida*.

*Toxin Formation.* Numerous unsuccessful attempts were made to demonstrate the presence of a toxin resembling that described by Pasteur (25).

Virulent bacilli were grown in infusion broth, with and without the addition of hemolyzed blood and fresh, unheated chicken tissue. The cultures were incubated aerobically and anaerobically for periods of eight hours to one week. The filtrates were tested by intravenous, intramuscular, and subcutaneous injections into chickens. Some filtrates were concentrated to one-tenth volume by distillation *in vacuo* and then tested. A further series of inoculations was made with bacilli dissolved in dilute alkali. In no instance did the inoculated animals die, nor symptoms develop resembling those of fowl cholera.

*Virulence.* From an epidemiological point of view, no property of bacteria is more important than that of virulence or pathogenicity. Hence in these studies an effort has been made to determine with care the disease-producing power of *P. avicida*.

The test animals were chickens from a single flock of healthy breeding hens, shipped to this laboratory one day after hatching and raised under uniform conditions. Twenty birds, four weeks old, weighing 100–150 gms., were used for each test. The bacterial cultures for testing were grown on hemolyzed blood agar for 15–18 hours, washed off, and suspended in infusion broth. The density of the suspension was standardized with the Gates' turbidometer (26) and brought to the concentration of an 18 hour culture. The dose employed was 0.2 cc., controlled by counting, and representing about 20,000,000 bacilli. The inoculum was deposited from a syringe fitted with a blunt pointed needle, on the intact nasal mucosa. The reasons for using the intranasal portal of entry are discussed in the following paper. After inoculation, each bird was kept in a separate cage. The tests extended over a ten day period, although deaths were infrequent after the fourth day.

To control the effect of variations in susceptibility of the chickens, one strain of "fluorescent" colony type organisms was reinjected into a control group of twenty birds with each test.

The results of these titrations are given in Table V, where "virulence" is expressed as percentage of birds dying of fowl cholera. Other determinations, if made on the identical culture, are included. The data indicate a distinct correlation between virulence and the attributes associated with colony form. Thus, the "blue" colony types proved relatively avirulent, while the "fluorescent" forms were highly pathogenic. Single tests on the "intermediate" colony strains gave

results varying from 0 to 60 per cent mortality. For this irregularity three explanations are possible;—technical difficulties, variations in the resistance of the birds, or actual differences in the virulence of the “intermediate” strains. Data to be presented in the succeeding

TABLE V

*Virulence and Other Properties of Pasteurella avicida of Different Colony Forms*

Colony form	Source	Strain	Acid agglutination (pH range)	Serum agglutination (Maximum dilution)	Virulence (Per cent mortality)
Fluorescent	Autopsy	629	2.4 only	0	10
		631	none	0	60 (av. 4)
		638	none	0	30 (av. 5)
Blue variant	Fluorescent	629 B	2.4–5.4	1280	0
		631 B	2.4–4.6	2500	0
		638 B	2.4–5.4	1280	0
Intermediate	Autopsy	729	3.0–4.2	2500	0
		730	3.0–4.2	2500	0
		731	3.0–4.2	1280	0
		733	2.4–3.5	320	0
		726	2.5–4.1	100	10
		798	2.5 only	0	0
		796	2.5–4.7	640	0
		635	3.0–5.4	1250	20
		641	2.4–5.0	1280	20
		642	2.4–5.4	640	60
		644	2.4–5.0	2500	20
		648	3.0–5.0	2500	40
Blue	Autopsy	651	3.0–5.0	5000	0
		647	2.4–5.0	640	0
		702	2.4–5.0	640	0
		773	2.4–5.4	5000	10
		779	2.5–5.2	640	0
		793	2.5–5.2	1250	0
		781	2.5–5.2	1250	20

papers render the latter possibility unlikely and indicate that strains of a given colony type are in general of similar virulence. The relative pathogenicity of “fluorescent,” “intermediate,” and “blue” colony types may be expressed for convenience as 50 per cent, 25 per cent, and 0 per cent respectively.

Attempts to raise virulence by growth in media, sera, and passage through fowls were not successful. "Blue" colony forms could not be changed to "intermediate" or "fluorescent," nor could an increase in virulence of any strain be demonstrated. Tests by means of host passage were made by intranasal instillations with and without the use of brilliant green (Bull, 27). One test at a season when titration mortality was low was carried on for seven passages (Table VI). The strain passed directly in blood from bird to bird showed no increase in virulence.

TABLE VI  
*Attempt to Increase Virulence by Chicken Passage*

Passage	Preliminary treatment of chicken	Survival time in days	Form of organism recovered	Virulence
I	Brilliant green intra-nasally	2	"Fluorescent"	
II	" "	12	"	
III	None	6	"	
IV	"	6	"	
V	"	3	"	
VI	"	3	"	5 chicks inoculated, 1 died.
VII	"			5 chicks inoculated, none died.

#### DISCUSSION

This study of 210 freshly isolated strains from known cases of fowl cholera has proved useful in defining and classifying the inciting microbe of fowl cholera, *P. avicida*. This definition and classification were made on the basis of general uniformity in morphology and growth characteristics in fluid media and like reactions in carbohydrates and indol production. By the aid of serological methods, a fundamental antigenic similarity of all strains of *P. avicida* has been determined. The extent of this specificity is, however, unknown. It may indicate antigenic identity of all strains or the presence of a common antigenic fraction among otherwise distinct serologic groups similar to the "protein" fraction of pneumococci (28). At present, however, further analysis is hindered by the apparent inagglutinability of certain otherwise typical strains.



However, a temporary classification of *P. avicida* is suggested on the basis of colony morphology. One type, "fluorescent," is associated with epidemics of fowl cholera, is highly virulent, stable in suspension, agglutinable only in very acid buffers, pH 2.4, and not at all in antisera. It resembles the "D" form of *P. lepiseptica* (29). The second type, "blue," is found in flocks where fowl cholera is endemic, is of low virulence, unstable in suspension, agglutinates in acid buffers over a wide zone, pH 2.4 - 5.6+, and in all *P. avicida* antisera. This type corresponds to the "G" form of *P. lepiseptica* (29). The third type, "intermediate," is associated with the more severe fowl cholera, and is "intermediate" in its behavior. This form bears some resemblance to the mucoid types of *P. lepiseptica* (29). These groupings have remained consistent throughout the present studies.

Hence there is available a definite criterion for the diagnosis of *P. avicida*, together with a sub-grouping of considerable epidemiological importance.

#### SUMMARY

1. A bacteriological study has been made of 210 fresh strains of Pasteurella obtained from typical cases of fowl cholera on seven widely separated poultry farms.

2. The strains have proved identical in consisting of small, pleomorphic, bipolar staining, Gram-negative, non-motile bacilli. They grew rapidly in infusion broth plus a trace of hemoglobin. They formed acid but no gas in media containing dextrose, saccharose, and mannite; indol was produced.

3. The strains fall into three distinct groups, according to their colony formation on hemoglobin agar. The "fluorescent" colony was large, whitish, opaque, exhibiting, under suitable conditions marked fluorescence. The "blue" colony was smaller, clear slate-blue, and non-fluorescent. The "intermediate" colony was moderately fluorescent at 15-18 hours growth, and "blue" thereafter. It was "blue" at all times when crowded and occasionally of "ring" form. "Fluorescent" colony cultures developed "blue" colony forms under certain conditions; otherwise all forms were stable.

4. Strains from "fluorescent" colonies were resistant to precipitation by acids, to sedimentation by centrifuging, and although they com-

bined with specific antiserum, did not agglutinate. They were relatively highly virulent and occurred in flocks where fowl cholera was epidemic.

5. Strains from "blue" colonies were precipitated by acids over a wide range of concentration and agglutinated strongly in antisera. They were relatively of low virulence and occurred in flocks where fowl cholera was endemic.

6. Strains from "intermediate" colonies varied in behavior between the "fluorescent" and "blue" strains. They came from a flock where fowl cholera was epidemic.

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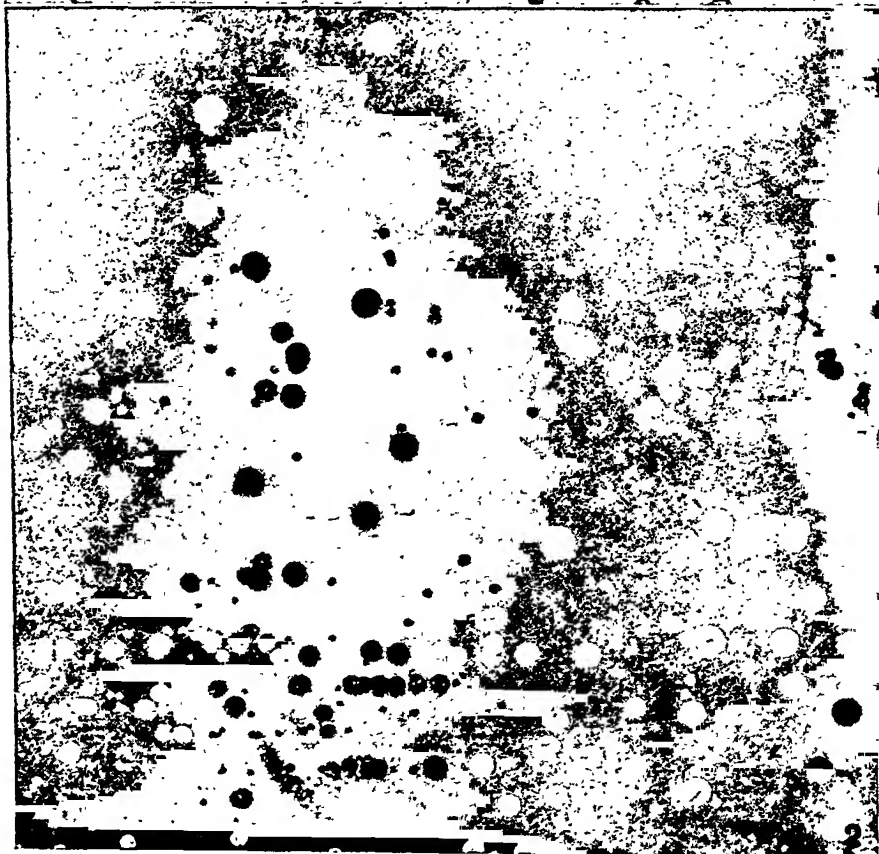
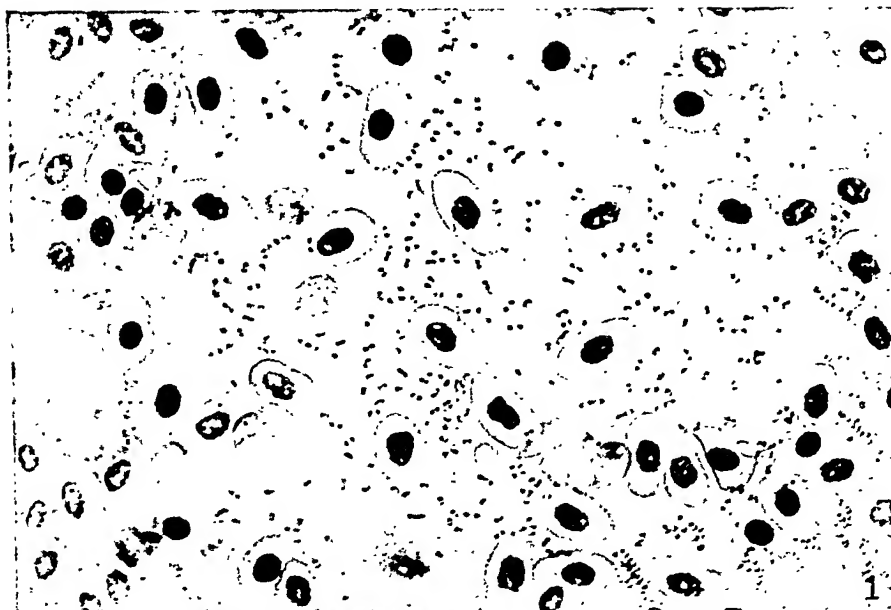
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## EXPLANATION OF PLATE 6

FIG. 1. *P. avicida* in blood of experimentally infected fowl. Antemortem specimen.

FIG. 2. "Fluorescent" and "blue" colony forms of *P. avicida*.





# THE EPIDEMIOLOGY OF FOWL CHOLERA

## III. PORTAL OF ENTRY OF *P. AVICIDA*; REACTION OF THE HOST

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### PLATE 7

(Received for publication, October 26, 1929)

The part of these studies dealing with the identification and characteristics of *P. avicida* has been reported in previous papers. It is proposed now to describe a number of tests made to determine: a) the normal portal of entry of these organisms into the chicken host, and b) the forms of infection so established.

Various portals of entry for the spontaneous infection have been suggested, but the gastrointestinal route has been considered as the most likely (Kolle u. Wassermann, III Aufl.). However, experimental infection by feeding methods is known to be difficult. Hertel (1) and Muller (2), for example, reported that when the inoculum is instilled directly into the esophagus of fowls and pigeons, in such a way as to avoid contact with the nasal and pharyngeal mucosae, or given in the relatively dry form of a pellet, infection does not take place. Nevertheless, the opinion of field workers that the disease is gastrointestinal in nature is generally accepted. The clinical course of acute fowl cholera and the pathological findings at autopsy are fully described in the literature (Kolle u. Wassermann, III Aufl.). But references to chronic cases are exceptional and the existence of localized infections is recognized only in occasional reports (3-9).

In contrast to this incomplete knowledge of *P. avicida* infection are the facts relative to infection by a similar organism, *P. lepiseptica* (10). These bacilli are known to enter the rabbit host by way of the upper respiratory tract and to induce various forms of localized and general disease.

Accordingly, tests were planned to determine whether the natural portal of entry of *P. avicida* into fowls was by way of the mouth or nasal passages, and whether in fowl cholera as in rabbit snuffles-pneumonia, infection appeared in various generalized and local forms.

*Normal Portal of Entry*

Preliminary tests showed that cultures of *P. avicida* administered *per os* to chicks and adult fowl did not infect, whereas when dropped into the nasal cleft, they brought about the typical disease (Table I-IV). The details of these tests are as follows:

Two fluorescent strains, "Pa" and "Kansas," described in the previous paper (11), were employed. The dose 0.2 cc., and the method for its control have been discussed (11). The birds were either five

TABLE I  
*Comparative per Os and Intranasal Injection of P. avicida into Fowl*

Test	Number of birds injected	Inoculum	Number dead	Per cent dead
I	15—adults	0.2 cc. "Pa" <i>per os</i>	0	0
	" "	0.2 cc. "Pa" intranasal	6	40
II	25—adults	0.2 cc. "Pa" <i>per os</i>	0	0
	" "	0.2 cc. "Pa" intranasal	7	28
III	12—pullets	0.5 cc. "Pa" <i>per os</i>	0	0
	" "	1 drop "Pa" intranasal	4	33
IV	20—adults	0.2 cc. "Kan" <i>per os</i>	0	0
	30— "	0.2 cc. "Kan" intranasal	15	50
V	25—chicks	0.2 cc. "Kan" <i>per os</i>	0	0
	50— "	0.2 cc. "Kan" intranasal	22	44
VI	20— "	0.2 cc. "Kan" <i>per os</i>	0	0
	" "	0.2 cc. "Kan" intranasal	11	55

weeks chicks, similar to those already described, or adult pullets from the same source (11). It is important to mention again that these fowls had had no previous exposure to *P. avicida*. Intranasal inoculations were made by holding the bird head downward and instilling the desired amount of inoculum from a syringe through a blunt needle over the surface of the mucosa on the roof of the nasal cleft. The *per os* instillations were made by placing the inoculum in a small gelatin capsule and introducing it at once into the esophagus of the chicken with a pair of forceps. After injection each bird was placed

in an individual cage and observed for ten days. Autopsies were performed on all fatal cases and cultures taken for identification.

Tests V and VI (Table I) were subjected to the following additional control, to demonstrate that cultures introduced in capsules were liberated and had retained their virulence. A number of additional birds were given the capsules, and every fifteen minutes thereafter one bird was killed, autopsied, and cultured. The specific strains of *P. avicida* were recovered from crop and upper intestinal tract of the autopsied bird, and then reinoculated intranasally in the usual manner into another series of normal chicks. These reinoculated strains were found to possess the same virulence as the control cultures. To prove that birds inoculated *per os* were as susceptible as those inoculated intranasally, they were reinoculated after ten days by the intranasal route. The mortality following the introduction of organisms by the nasal route was similar to that of the control intranasal series. It was concluded from the tests that *P. avicida* does not infect by way of the gastrointestinal tract. On the other hand, the chickens which succumbed to the intranasal instillations presented the usual clinical and pathological lesions typical of fowl cholera. This was regarded as evidence that the natural portal of entry of *P. avicida* is by way of the upper respiratory tract.

#### *Reaction of the Host*

To gain more information on the mode and route of infection of *P. avicida*, and especially to study the varieties of host response, series of birds were given intranasal doses of the organisms.

The first tests were made to determine the extent of variation in per cent mortality of groups of similar birds given similar doses of the same strain of *P. avicida*.

"Fluorescent" colony strains, "Pa," "638," and "Kansas," and "intermediate" colony strains "629" and "631" were prepared as usual (11) and instilled intranasally in standard doses, 0.2 cc., 20,000,000 bacilli. The birds employed were 4-5 weeks old, 100-150 gms. chicks, raised under uniform conditions and free of previous exposure to *P. avicida*. At least twenty were used for each titration. The results of these tests are given in Table II.

Nine titrations of the "Pa" strain showed an average mortality of  $31.9 \pm 2.6$  per cent. This average figure does not include the two



low values of May 16 and 23. Three titrations of "638,"  $40 \pm 4$  per cent; nine of "Kansas,"  $50.5 \pm 5$  per cent; three of "629,"  $16 \pm$

TABLE II  
*Comparison of Results of Repeated Virulence Tests on Same Strains*  
(5 weeks chicks; dose 0.2 cc. culture, intranasally)

Date	Strain	Number injected	Per cent mortality
March 29, '27	"Pa"	63	35
April 4		21	29
11		21	33
18		27	29
May 3		56	31
5		50	30
9		66	29
16		22	14
23		30	13
Oct. 27, '28		10	40
Dec. 8	629	13	31
Feb. 6		10	10
March 10		10	20
25		10	20
March 8	631	10	40
21		20	35
April 11		10	60
May 2		20	35
Jan. 29	"Kansas"	10	60
Feb. 4		50	44
6		30	50
8		20	65
14		35	46
April 24		10	40
27		10	50
28		10	50
May 7		20	50

\* This average does not include the low determinations of May 16 and 23, 1927.

4.5 per cent, and four of "631,"  $42.5 \pm 8.7$  per cent. The small variations in per cent mortality between tests indicate that the technique was adequate, and that under these conditions the virulence of

each strain was constant and the average resistance of the groups of chicks the same. Tests with adult birds gave similar results.

The possible effect of season is shown in the mortality figures of the "Pa" series. The drop in death rate in May from a relatively constant level of  $30 \pm$  to 15 per cent, and the subsequent rise to this level again in October is a phenomenon similar to those occurring in mouse typhoid, mouse pneumonia, and rabbit pneumonia infections (12).

The effect of dosage on mortality is shown in Table III. For this test, each of six groups of ten birds were given, in uniform volume, a definite dose of *P. avicida*, "Kansas," ranging from ten times the standard amount of 20,000,000 bacilli to 1/10,000 of this amount. The resulting mortality indicates that groups of chicks react similarly

TABLE III  
*Effect of Varying Dosage on Mortality*  
(0.2 cc. "Kansas" strain, intranasally, into groups of 10 chicks)

Dose	Per cent mortality
Standard (equal to 18 hr. broth culture).....	40
10 times standard.....	80
Standard diluted 1:10.....	50
" " 1:100.....	40
" " 1:1000.....	40
" " 1:10,000.....	40

to doses between 20,000,000 and 20,000 bacilli, but are more susceptible to greater amounts and less susceptible to smaller numbers. Two additional tests of this sort gave similar results.

The response of young birds to the intranasal instillations of *P. avicida* took two forms,—either they died abruptly of typical fowl cholera, or remained quite healthy. None showed chronic or local forms of infection. Nevertheless, evidence was obtained indicating that *P. avicida* infection in nature and under proper experimental conditions takes the form not only of acute septicemic fowl cholera, but also of chronic and localized disturbances.

Indirect evidence of local and of chronic *P. avicida* infection came in part through the kindness of Dr. Arnold Branch and William Steenken, Jr., and is presented with their permission at this time.

A small flock of nineteen fowl maintained at Trudeau for studies on avian tuberculosis became infected "spontaneously" with *P. avicida*. Within a few

TABLE IV

*P. avicida* Infection in a Flock of Chickens at Trudeau, N. Y.

No.	Course of disease	Autopsy findings		Occurrence of <i>P. avicida</i>				
		External	Viscera	Blood	Wattle	Roup	Eye	Pericardium
A 854	Acute		Pericarditis	+				+
C 33	Acute		Liver degeneration	+				
A 861	Acute		Liver degeneration	+				
A 853	Acute	Caseous wattle	Pericarditis	+				+
			Liver degeneration					
C 34	Acute	Wattle disease	Pericarditis	+	+			+
A 858	Acute	" "	Pericarditis	+	+			+
			Pneumonia					
A 852	Acute	Wattle disease; roup	Focal hemorrhage in lung	+				
C 31	Acute		Pericarditis	+				
A 863	Acute	Wattle disease	Pericarditis	+				+
			Perihepatitis					
C 32	Acute	" "	Caseous lung focus	+	+			
B 219	Chronic	Rhinitis; roup		+		+		
B 218	Chronic	Roup		0		+		
E 31	Chronic	"		+		+		
B 222	Chronic	Roup; intraorbital abscess		0		+	+	
E 32	Chronic	Roup		0				
B 217	Chronic	Roup; rhinitis	Caseous nodules in liver	0		+		
B 220	Chronic	Roup		0		+		
B 223	Chronic	"		0				
B 221	Chronic	"		0				

days 50 per cent died of typical fowl cholera and were autopsied. Positive blood cultures were obtained in all cases. Six of the number showed localized wattle

disease and one roup. Cultures taken from three of the infected wattles yielded the epidemic strain of *P. avicida*. Nine of the birds survived but not without sickness and loss of weight. After a few weeks they were killed and autopsied. Two had positive blood cultures. All showed severe upper respiratory lesions, manifested clinically as mucopurulent discharge, roup, rhinitis, and orbital abscess. From the lesions of six of these cases, *P. avicida* was recovered. One bird showed a chronic pulmonary lesion; another a similar focus in the liver. The strains sent to the Rockefeller Institute laboratory appeared identical; no distinction was possible between cultures from local lesions of chronic cases and blood cultures of acute "cholera" cases.

An additional study of "spontaneous" local lesions was made on birds from poultry farms in New Jersey. Lesions from twenty-seven

TABLE V

*Bacteriological Examination of Local Infections Occurring in Chickens (a) "Spontaneously" and (b) Following Intranasal Inoculation with P. avicida*

Lesion	From "spontaneous" lesions		From lesions occurring in experimentally inoculated birds	
	Positive for <i>P. avicida</i>	Negative for <i>P. avicida</i>	Positive for <i>P. avicida</i>	Negative for <i>P. avicida</i>
Ocular roup.....	7	3	7	0
Mixed ocular and sinus roup.....	0	1	0	0
Sinus roup.....	0	5	4	1
Edema of wattle.....	5	3	1	1
Otitis media.....	2	0	8	0
Submandibular abscess.....	1	0	0	0

birds on a number of different farms were cultured; fifteen yielded *P. avicida* of the "blue" colony type (Table V) (Figs. 1 to 3). Of these, nine were tested for virulence and, as usual, failed to kill.

Direct evidence of chronic and localized *P. avicida* infection was obtained by giving cultures intranasally to tested non-carrier, healthy fowl. In all, twenty-two local lesions occurred, classified as roup, edema of wattle, and abscess. Twenty of these proved positive (Table V). It is concluded, therefore, that roup, edema of the wattle, and sinusitis in fowl are frequently local forms of *P. avicida* infection.

One other form of *P. avicida* infection was studied,—the carrier

state. That a certain number of birds in infected flocks carry the organisms in the nasal cleft will be brought out in the following paper; at this time it will suffice to present the results of tests on birds given the organisms experimentally by the nasal route. Birds were tested before inoculation and at intervals thereafter (Table VI). The results show that the carrier state is a definite, though clinically

TABLE VI  
*Occurrence of Carriers among Injected Birds*

Date	Strain	Number injected	Per cent mortality	Per cent carrier
April 4	642	10	30	0
February 7	642	10	60	0
	648	10	40	0
March 8	631	10	40	10
	635	10	30	10
	638	10	50	10
	651	10	50	0
	661	10	30	0
	667	10	40	0
	672	10	40	0
April 11	631	10	60	0
April 18	661	10	30	0
April 5	660	10	0	20
	690	10	0	30
	735	10	0	10
	793	10	0	10
	629 B	10	0	20
March 22	745	10	0	20
	749	10	0	10
	773	10	0	20
March 29	770	10	10	30
	779	10	0	20
	785	10	10	10

concealed form of *P. avicida* infection, and that it occurs most frequently with the "blue" type, so-called endemic forms, and rarely with the "fluorescent" colony "epidemic" types.

#### DISCUSSION

The data contained in this report indicate that fowl cholera is not primarily a gastrointestinal disease, but that, like rabbit pasteurellosis

(12), it is upper respiratory in origin. These observations show further that the etiological agent, *P. avicida*, gives rise not only to the well-known septicemic cholera, but also to various types of chronic pneumonia and liver abscesses, and local disturbances of the upper respiratory tract, such as roup, sinusitis, and inflammation of the wattle. Still another form of infection, clinically unrecognizable, is the "healthy" nasal-carrier state. Finally, it appears that of any group of healthy, selected chickens given a similar dose intranasally, some will resist infection entirely.

The fact that chickens under controlled conditions react differently to the same intranasal dose of *P. avicida* indicates that they differ in their ability to resist the infection and that, as in the case of rabbits exposed to *P. lepi-septica* (13), the most resistant individuals are not infected; those less so become carriers, others more susceptible contract local upper respiratory lesions, while the most susceptible die of the acute generalized infection.

Another observation of importance is that a given strain of *P. avicida* given intranasally to a number of groups of controlled and selected birds gives rise, in general, to a similar amount of mortality. From this the conclusions are drawn that the *technique* of titration is adequate for measuring virulence of organisms, and that under these conditions the *virulence* of a given strain and the *average resistance* of the chickens are constant. The summer decrease in mortality is at present considered due to an increase in the resistance of the birds.

The standard dose, estimated to be approximate to that available to flocks at epidemic times, may be increased ten times, or decreased 1000 times without apparent effect on mortality. Outside of those limits, however, dosage influences mortality. These observations are in accord with similar tests with mouse Friedländer (14) and typhoid (15) bacilli.

#### SUMMARY

1. In the experiments here presented, *P. avicida* proved incapable of inciting fowl cholera when introduced directly into the alimentary tract. On the other hand, when administered into the upper respiratory passages, it induced typical disease.

2. When *P. avicida* was introduced into the nasal passages of con-

trolled, selected chickens, some died of typical septicemic cholera, a few developed chronic pneumonias and other conditions and succumbed, a few developed localized upper respiratory inflammations, such as rhinitis, roup, and wattle involvement, while yet a few others became "healthy" nasal carriers. Usually, however, more than 50 per cent resisted infection. Repeated titrations of this sort gave, in general, uniform results, save that in spring and summer the per cent mortality decreased.

3. *P. avicida* was recovered from a number of cases of "spontaneous" roup, rhinitis, and wattle disease.

4. Groups of chickens reacted similarly to doses of virulent *P. avicida* varying from 20,000,000 to 20,000. Outside these limits, dosage exercised a marked influence on mortality.

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#### EXPLANATION OF PLATE 7

Spontaneously occurring lesions yielding *P. avicida* on culture.

FIG. 1. Wattle disease.

FIG. 2. Ocular roup. The lesions on the comb are due to fowl pox.

FIG. 3. Sinus roup.







# THE EPIDEMIOLOGY OF FOWL CHOLERA

## IV. FIELD OBSERVATIONS OF THE "SPONTANEOUS" DISEASE

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The results of special studies on the bacteriology of fowl cholera, its mode of infection, and reaction of the host have been dealt with in the preceding papers (1 a, b). It is proposed now to present the data collected from field studies as the disease occurs spontaneously in poultry flocks.

### *Methods*

A series of expeditions was made to the various poultry farms chosen for study. At each trip, a census was taken, the amount of clinical infection noted, and cultures made from the nasal passages of all or part of the flock. These cultures were then brought to the laboratory at New Brunswick or New York for study. In addition to this, birds dying within the period of observation were sent to New Brunswick if possible, and there autopsied and cultured.

To test for carriers, sterile cotton swabs of appropriate size were passed over the nasal mucosa and streaked on hemolyzed blood agar contained in 150 cm. Petri dishes. After 18-24 hours' incubation, the number of colonies of *P. avicida* on the plates was estimated and suspicious colonies transferred to appropriate media for identification and colony type determination. Cultures showing characteristic colony appearance and morphology, acid production in dextrose, saccharose, and mannite, and a positive indol test were considered *P. avicida*.

Individual birds in the flock were identified by numbered leg bands.

*A Study of Epidemic P. avicida Infection*

This investigation was initiated by the bacteriological examination of three dead birds sent to New Brunswick by their owner at Merrick, N. Y. He reported that the death rate at his farm had been consistently low, but that suddenly an increase had taken place.

The poultry plant was found to consist of three widely separated houses, well-kept and clean, large and airy. One entire side of each unit was enclosed by glass. The diet was carefully supervised and contained an abundance of sprouted oats and cod liver oil.

House I, 92 x 16 ft., contained 425 White Leghorn pullets, December, 1927. Cholera then appeared and gave rise to a relatively low constant death rate. By March, about 35 per cent of the population had succumbed. The entire population of 341 individuals was tested

TABLE I

*Incidence of P. avicida Carriers and Occurrence of Localized Lesions at Merrick, Long Island*

House	Date	Number of fowl examined	Per cent carriers	Nasal catarrh	Canker	Roup	Swollen wattles
II	Jan. 25 '28	22	45	0	0	0	0
I	Jan. 28	341	11	25	0	1	13
I	May 10	90	1	9	0	1	1

for carriers late in January, 1928. Thirty-seven or 11 per cent were positive, and note was made of a high incidence of local infection (Table I). Late in March, the survivors were combined with a similar group from House III. In May, 1928, a sample of 90 birds was tested for carriers. Only one was positive, although six of the sample were known to have been carriers at the previous testing in January. The physical condition of the birds also seemed much improved (Table I).

House II, 100 x 20 ft., housing 700 White Leghorn pullets, suffered the worst outbreak. The disease appeared here also in December, 1927, but in a more severe form. In four weeks 45 per cent of the population had succumbed. Losses then ceased abruptly. Late in January, when a "sample" of twenty-two birds was tested for carriers, 10 or 45 per cent were positive, but no local infections were noted.

House III was divided into three compartments. The first, 65 x 22 ft., housed 400 White Leghorn hens, the second, 50 x 22 ft., contained 300 similar hens. These two populations remained free of cholera. The remaining end compartment, 40 x 22 ft., housed 300 White Leghorn pullets, similar to those occupying Houses I and II. These birds began to die after mortality in Houses I and II had practically ceased, and by late spring the total deaths were estimated to be 37 per cent. No carrier determinations were made at the time of the epidemic, but the tested sample of survivors from Houses I and III showed a very low rate.

The total mortality in this flock can merely be estimated. The owner stated that 1325 pullets were added to his stock in the summer preceding the epidemic. Of these, 525 were known to have died during the winter of 1928. Forty-seven were received at the laboratory for autopsy; all but one yielded pure cultures of *P. avicida*. It was presumed, therefore, that the deaths were in the main due to *P. avicida* infection.

Of the strains of *P. avicida* recovered, three were of the "fluorescent" and 64 the "intermediate" colony type. No "blue" colony forms were obtained. The three "fluorescent" colony strains came from the blood of fatal cases in House II at the height of the epidemic. The range of acid agglutination was tested for 48 of the strains plus three "blue" colony variants obtained from the "fluorescent" forms. The results are given in Table II. The three "fluorescent" strains showed the typical narrow zone of flocculation, pH 2.4-3.0; the "blue" variants, the usual wide zone, pH 2.4-5.4, and the "intermediates," a scattered range between the limits, tending toward the more acid range. No distinction between "autopsy" and "carrier" strains was apparent.

Agglutination tests were made with "Pa" antisera (1 a) diluted 1:100. All save the three "fluorescent" colony forms showed flocculation. The agglutination titre of twenty-eight "intermediate" plus the three "blue" variants from the "fluorescent" strains was then determined (Table III). One "blue" variant agglutinated to 1:640; the others approximately to titre. Agglutination of the intermediates ranged from 1:160 to 1:2500; the greater number fell in the range 1:640-1:1280.

The virulence of fourteen autopsy strains and nineteen carrier strains was tested according to the method described in the second paper of this series (1 b). Twenty birds were used for the tests above, for each strain. The results are recorded in Tables IV and V. Several of the autopsy strains were titrated repeatedly. Hence the data on these strains are considered to be the more accurate index of virulence. The average mortality of all autopsy strains was 33 per cent.

TABLE II

*Zone of Acid Agglutination. P. avicida Strains from Merrick, N. Y.*

Colony type	Zone of agglutination pH range	Number of strains
Fluorescent	2.4 only	1
	2.4-3.0	1
	3.0 only	1
Blue variant	2.4-5.0	2
	2.4-5.4	1
Intermediate	2.4-4.2	2
	2.4-5.4	4
	2.5-3.4	2
	2.5-3.6	3
	2.5-4.1	3
	2.5-4.4	4
	2.5-4.7	3
	2.5-5.1	5
	3.0-3.5	1
	3.0-4.2	1
	3.0-5.0	9
	3.0-5.2	2
	3.0-5.4	1
	3.2-3.5	1
	3.4-4.7	3
	Spontaneous	1

TABLE III

*Reaction with "Pa" Type Serum. P. avicida Strains from Merrick, N. Y.*

Colony form	Agglutination Maximum serum dilution	Number reacting
Fluorescent	None	3
Blue variant	1:640	1
	1:1280	1
	1:2500	1
Intermediate	1:160	1
	1:320	2
	1:640	7
	1:1280	10
	1:2500	8

TABLE IV  
*Virulence of Merrick "Autopsy" Strains of P. avicida*

Culture	Type	Isolated	Tested	Per cent dead	
629	Fl.	Jan. 22	Jan. 23	20	av. 17
			Feb. 15	20	
			Mar. 6	10	
630	Int.	Jan. 22	Jan. 23	15	av. 17
			Mar. 8	20	
631	Fl.	Jan. 22	Jan. 23	35	av. 44
			Feb. 8	40	
			Mar. 8	40	
			Apr. 11	60	
635	Int.	Jan. 30	Mar. 8	30	
638	Fl.	Jan. 30	Feb. 15 ('28)	60	av. 40
			Mar. 8	50	
			Mar. 21	40	
			Apr. 18	30	
			Apr. 25	40	
			Mar. 6 ('29)	30	
641	Int.	Jan. 30	Mar. 6	20	
642	Int.	Jan. 30	Mar. 6	60	av. 45
			Apr. 5	30	
644	Int.	Jan. 30	Mar. 6	20	
645	Int.	Feb. 1	Feb. 15	50	av. 55
			Feb. 20	60	
647	Int.	Feb. 1	Mar. 6	20	
648	Int.	Feb. 1	Mar. 6	40	
650	Int.	Feb. 1	Feb. 15	30	av. 40
			Feb. 20	50	
651	Int.	Feb. 1	Mar. 8	50	
702	Int.	Feb. 6	Mar. 6	20	
Average.....				33%	

In these titrations no significant difference in the killing power of "fluorescent" and "intermediate" colony strains was apparent. The average mortality of all "carrier" strain titrations was 21 per cent. This figure is lower than that of the "autopsy" strains, but since only one titration of each was made, the difference is of questionable significance.

The interpretation of these studies is necessarily limited. However, the following summary includes the salient facts. An epidemic of fowl

TABLE V  
*Virulence of Merrick "Carrier" Strains of P. avicida*

Culture	Type	Isolated	Tested	Per cent dead
687	Intermediate	January 28	February 20	20
660	"	" 28	April 5	0
690	"	" 28	" 5	0
654	"	" 22	" 11	50
655	"	" 22	" 11	0
657	"	" 22	" 11	20
658	"	" 22	" 11	50
653	"	" 22	" 18	20
659	"	" 22	" 18	20
661	"	" 22	" 18	30
663	"	" 22	" 18	20
664	"	" 22	" 18	10
665	"	" 22	May 2	0
666	"	" 22	" 2	30
667	"	" 22	" 2	40
668	"	" 22	" 2	30
669	"	" 22	" 2	30
670	"	" 22	" 2	10
672	"	" 22	" 2	40
Average.....				21

cholera arose during the winter in a healthy flock of birds. Its mode of onset was not determined, and the owner stated that his birds had previously been free of the disease. The epidemic ran different courses in three population units and failed to appear in a fourth group of hens. In one infected group, the epidemic took an explosive form, with high, wave-like mortality over a brief period. Few cases of localized infection developed. In two other groups, the epidemic smoldered with low, relatively constant mortality rate over a period of

months. Strains of *P. avicida* from the two populations examined were indistinguishable. Following the epidemics, carriers were very few in number.

From these facts, it seems probable that "epidemic" strains of *P. avicida* were introduced into the flock from some outside source. Apparently, the birds possessed no specific immunity to the infection, but those in House I may have been more resistant than the population of House II. The crowding in House II, by decreasing the resistance of the inhabitants and increasing the risk of infection, may have been responsible for the explosive type of attack. Finally, the fact that few post-epidemic carriers were encountered indicates that, as in similar "D type" rabbit infections (1 c), "epidemic" strains of *P. avicida* are relatively non-vegetative.

### *B. Study of Endemic P. avicida Infection*

A similar investigation was made at a small farm in Deans, N. J., consisting of 45 White Leghorn hens, cared for entirely by the owner. The pen was very dirty and neglected, dark, and in an exposed location. Some sort of epidemic of fowl cholera was known to have occurred in previous years, and the birds under observation were known to be survivors.

Five trips were made to the farm, November, 1927 to March, 1928. A census was taken, the clinical condition of the birds noted, and cultures obtained from the nasal passages of each. The birds were tagged, so that a four months' record of each was obtained. The results of these investigations are given in Table VI.

No birds died within the period. Nineteen showed local upper respiratory infections—roup, canker, mucous discharge, wattle disease—at one or more examinations. Thirty-one proved to be carriers of *P. avicida* on at least one test. The carrier rates at each examination were 9.7, 40.5, 16.2, 31.5, and 29.0 per cent.

Fifty-two strains of *P. avicida* were recovered from this flock. Apparently, all formed "blue" colonies (1 a) on the first transfer. Twenty-seven were titrated in "Pa" antiserum (1 a). The results are shown in Table VII. Two did not agglutinate; two showed spontaneous agglutination; three reacted within the range 1:32-1:128; four agglutinated at a dilution of 1:256; fourteen to 1:152, and two to 1:1024 and 1:2048 respectively.



TABLE VI

Results of Serial Examinations for *P. avicida* Carriers, at Deans, N. J.

Bird	November 23	December 17	January 17	February 7	March 23
1	0	0 Canker	0	—	+
2	0	0 Mucus	0	0	0
3	—	0	—	0	+
4	+ Canker	0	0	0	+
5	0	+ Mucus	0	0	0
6	0	+	0	0	+
7	0 Membrane	—	0	+ Canker	0
8	0	0 Canker	0	+	—
9	0	0 Mucus	0	0 Roup	—
10	0	0	0	0	0
11	0	0 Mucus	0	+	0
12	+	+ Mucus	0	0	—
13	0	+ Wattle	0	0 Mucus	0
14	0	+ Canker	0	+	+
15	0	0	0	0	0
16	0	0	0	0	0
17	0	+	0	—	0
18	0	+ Mucus	0	+ Mucus	0 Roup
19	+	+	+	0	+
20	—	0 Mucus	0	+	0
21	0	+	0	0	0
22	0	0	0	0 Mucus	0
23	0	+ Canker	0	0 Roup	0
24	0	—	0	0 Roup	—
25	0	0	+	0	0
26	0	0	0	0	0
27	+	0	0	+	0
28	0	+	+	+	+
29	0	0	0	0	0
30	0	0	0	0	0
31	0	0	0	0 Canker	0
32	0	+	0	0	—
33	0	+	0	0	0
34	0	+	+	0	0
35	0	0	0	+	0
36	0	0 Mucus	0	0 Mucus	0
37	0	0	+	+	0
38	0	0	0	+	+
39	0	0	0	0	—
40	0	+ Mucus	0	—	0
41	0	0	0	0	0
42	0	0	0	0	+
43	—	+	0	—	+
44	0	+	+	+ Roup	+
45	—	—	—	+	—
Positive	4 = 9.7%	17 = 40.5%	6 = 16.2%	13 = 31.5%	11 = 29%
Negative	37	25	37	28	27
Not done	4	3	2	4	7

0 = Negative; + = *P. avicida* recovered; — = No examination.

Acid agglutination tests, made on twenty-nine strains, are given in Table VIII. Twenty-four showed the wide zone characteristic of "blue" colony forms, but five came down well toward the acid side, pH 2.4-4.0

This study may be summarized as follows: in a small flock, known to have survived cholera infection the previous year, the endemic manifestation of the disease was studied. No deaths occurred, but the per cent of local lesions and nasal carriers increased during the winter

TABLE VII

*Reaction with "Pa" Serum. P. avicida Strains from Deans, N. J.*

Serum dilution	Number of strains reacting	Serum dilution	Number of strains reacting
No reaction	2	1:256	4
Spontaneous	2	1:512	14
1:32	1	1:1024	1
1:64	1	1:2048	1
1:128	1		

TABLE VIII

*Zone of Acid Agglutination. P. avicida Strains from Deans, N. J.*

Zone of agglutination pH range	Number of strains	Zone of agglutination pH range	Number of strains
2.4 only	1	2.4-4.6	4
2.5-3.0	1	2.4-5.0	1
2.4-3.2	1	2.4-5.4	6
2.4-3.5	1	3.0-4.2	1
2.4-4.0	1	3.0-4.6	3
2.4-4.2	5	3.0-5.4	4

months. Apparently the birds differed in their ability to resist infection, for in spite of the evidence of spread of the organisms, some birds remained free throughout. All, however, were probably more than normally resistant, for they were survivors of a previous epidemic. The organisms recovered possessed the general characteristics of the "blue" colony form, although a few resembled the "intermediates" in their behavior. Unlike the "epidemic" fluorescent and intermediate colony forms, they exhibited the power of vegetation and spreading in the community. In this respect they were similar to the "mucoid" strains of *P. leipseptica* (1 c).

## SUMMARY

Field studies of fowl cholera on two commercial poultry farms are described. One farm, previously free of cholera, was studied during an active epidemic, which occurred during the winter months. The strains of *P. avicida* recovered, both from "autopsy" and from "healthy carriers" proved generally similar, and to be of the "fluorescent" or "intermediate" colony type, which is of relatively high virulence. After the subsidence of the epidemic, these strains tended to disappear.

The second flock consisted of a small group of birds which had survived an epidemic of cholera the previous year, and in which the infection was prevailing in endemic form. No deaths occurred during the period of observation, but the number of birds with localized lesions and the number of carriers increased to a high level during the winter months. The strains of *P. avicida* were apparently of the "blue" colony form, although some, as shown by their acid and serum agglutination reactions, resembled the "intermediates." These strains appeared to be spreading rather than dying out. The individual fowls differed in their response to the presence of infection; some showed localized lesions, others were carriers, while still others seemed entirely refractory.

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## THE EPIDEMIOLOGY OF FOWL CHOLERA

### V. FURTHER FIELD OBSERVATIONS OF THE SPONTANEOUS DISEASE

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Field studies of the sort described in the previous paper (1b) were made on one additional poultry farm at Belle Meade, N. J., where fowl cholera was known to have prevailed in endemic form. The observations at this farm were better controlled, more detailed, and extended over a longer period of time. The purpose of this study was twofold: 1) To gain further knowledge of endemic cholera, the common, almost ubiquitous form of the disease in this locality, and 2) To test a method of control. The results obtained are described in this paper.

#### 1926-1927

The flock at Belle Meade consisted of some 2000 White Leghorn chickens, maintained for commercial purposes. Winter outbreaks of fowl cholera were known to have occurred in 1925-1926. The farm was of modern construction and well-kept, comprising a long building with subdivisions, several outbuildings, and unrestricted "runs." Windows extended along one entire side of the large building. Details of the floor plan are given in Text-Figure 1.

*House I* contained 239 pullets. Seven tests for carriers were made between March 4 and May 17, 1927, on random samples of four to eighty-four birds (Table I). No carriers were detected, but some individuals were not examined. Twenty-two birds died in this house from December, 1926 to June, 1927. All were autopsied and four showed *P. avicida* infection (Table II).

*House II* contained 273 pullets. Six tests for carriers were made from March 4 to May 4, 1927, during which time each bird received at least one examination (Table I). No carriers were found. Eleven deaths occurred during the period December, 1926—June, 1927. They were autopsied, but proved negative for *P. avicida* infection (Table II).

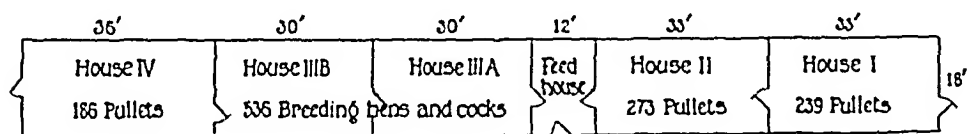
*House III* was divided into two compartments prior to January 1, 1927. One compartment contained pullets; the other, hens which had survived a fowl cholera

epidemic the previous winter. In January, the pullets were removed to House IV, the partition removed, and a number of cockerels added. This group of hens and cockerels composed the breeding stock.

The entire group of 536 was tested for carriers during the period January-March, 1927, the greater number receiving the examinations (Table I). Seventy-four carriers of *P. avicida* were found. Eighty-four deaths occurred during the year, of which sixty-one were shown at autopsy to be due to fowl cholera (Table II).

House IV contained 186 pullets, 85 of which were transferred from House III. All were tested at least once, and most of them twice. Ten carriers of *P. avicida* were found (Table I). During the year twenty-five died and were autopsied. Eighteen had fowl cholera and yielded positive cultures (Table II).

These observations permit a tentative explanation of the mode of spread of the disease during the year. In the first place, the infection was brought to the flock, in all probability, by the hens in House III,



TEXT-FIGURE 1

themselves survivors of an outbreak the year before, and known to have been infected early in the fall (Table I). Its spread was brought about by the pullets in House III, which were later transferred to House IV, and by way of an occasional straggler gaining access to Houses I and II. The infection assumed a seasonal character, but the septicemic form of the disease and the carrier state increased in amount during the winter months, and practically disappeared during the summer (Text-Figure 2). Finally, there proved to be a distinct correlation between the number of *P. avicida* carriers and the number of deaths from fowl cholera. In House II, for example, where each bird was examined and found negative for *P. avicida*, there was no cholera mortality. In House I, where the carrier incidence, if any, must have been low, specific deaths were low. In Houses III and IV, on the contrary, both the carrier incidence and cholera death rate were high, the latter representing the greater part of the total mortality in these houses (Text-Figure 3).

TABLE I

*Carrier Tests on "Belle Meade" Poultry Flock, 1926-1927*

Date	Number of birds examined				Carrier percentage			
	House I 239 birds	House II 273 birds	House III 536 birds	House IV 186 birds	House I	House II	House III	House IV
December 1	0	0	9	90	—	—	0.0	0.0
January 5	0	0	222	3	—	—	6.3	0.0
January 11	0	0	213	0	—	—	5.3	—
January 24	0	0	212	4	—	—	20.3	0.0
January 31	0	0	223	2	—	—	0.4	0.0
February 7	0	0	119	0	—	—	5.1	—
March 4	38	36	9	42	0.0	0.0	2.4	0.0
March 14	34	56	0	47	0.0	0.0	—	4.2
March 29	67	38	0	45	0.0	0.0	—	11.0
April 12	84	23	0	0	0.0	0.0	—	—
April 26	80	48	0	0	0.0	0.0	—	—
May 4	4	67	0	45	0.0	0.0	—	6.6
May 17	15	0	0	0	0.0	—	—	—
June 3	0	8	0	0	—	0.0	—	—
Total.....	322	276	1007	278				

0 = No test.

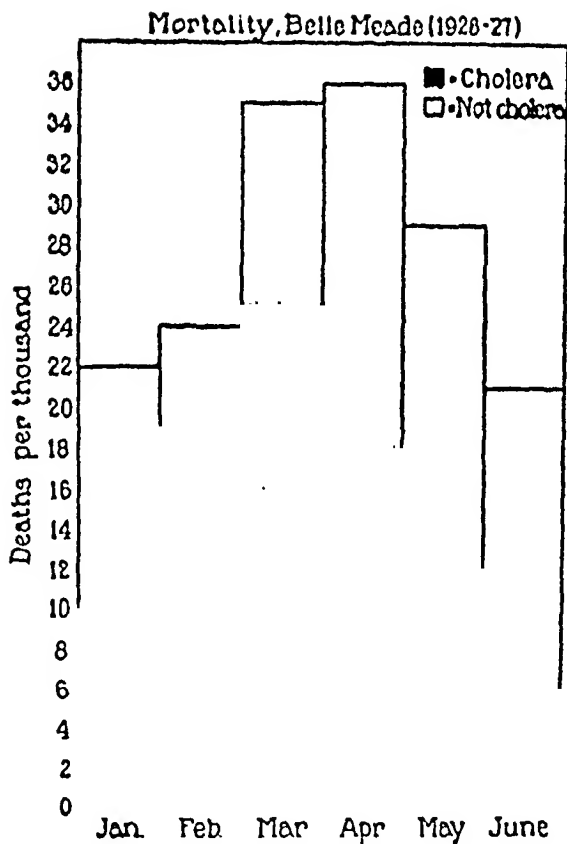
— = No test.

0.0 = Zero per cent carriers.

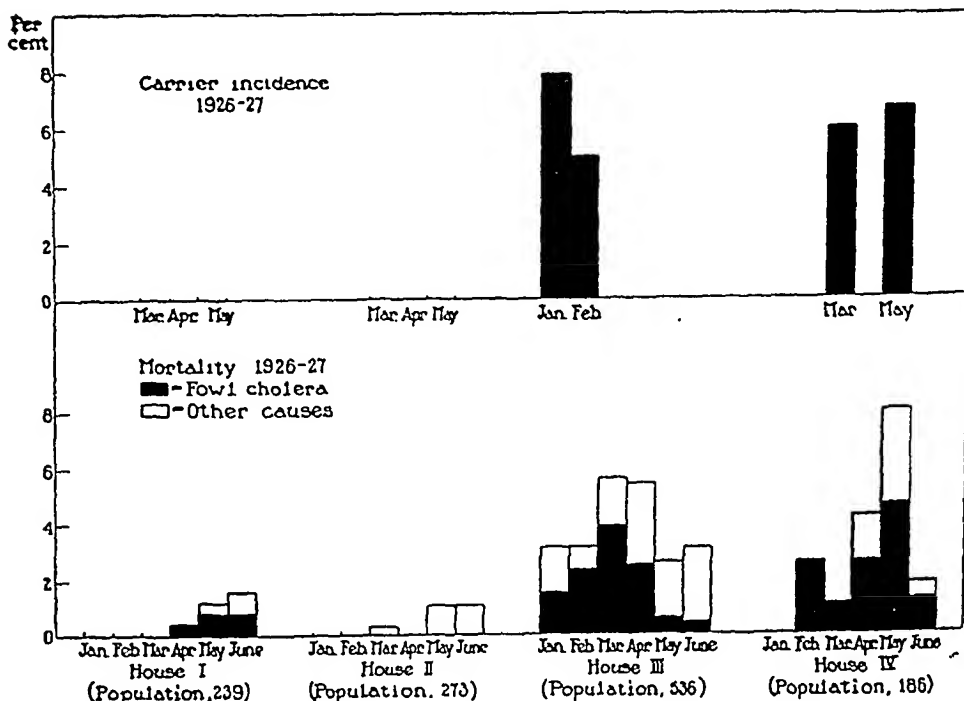
TABLE II

*Mortality of "Belle Meade" Poultry by Months, 1926-1927*

Month	House I		House II		House III		House IV	
	Fowl cholera	Other causes	Fowl cholera	Other causes	Fowl cholera	Other causes	Fowl cholera	Other causes
December	0	0	0	0	0	0	0	0
January	0	0	0	0	8	9	0	0
February	0	0	0	0	12	4	5	0
March	0	0	0	1	20	9	2	0
April	1	0	0	0	12	14	5	3
May	2	1	0	3	3	9	8	5
June	1	1	0	3	2	12	2	1



TEXT-FIGURE 2



TEXT-FIGURE 3

## 1927-1928

During the second year, the work was carried on as before, except that an attempt was made to remove all carriers from the breeding population and test the effect of this procedure on the amount of fowl cholera. As a preparation for this

TABLE III

*Results of Carrier Tests; Population A, Belle Meade, N. J., 1927-1928*  
Population = 396

Date	Number examined	Number of carriers	Per cent carriers
11/ 2/27	31	0	0.0
9	196	0	
30	30	0	
12/14	28	0	0.0
21	21	0	
29	60	0	
1/ 8/28	60	0	0.0
2/29	50	0	0.0
3/21	66	0	0.0
4/11	54	0	0.0
5/ 2	139	0	0.0

TABLE IV

*Results of Carrier Tests; Population B, Belle Meade, N. J., 1927-1928*  
Population = 714

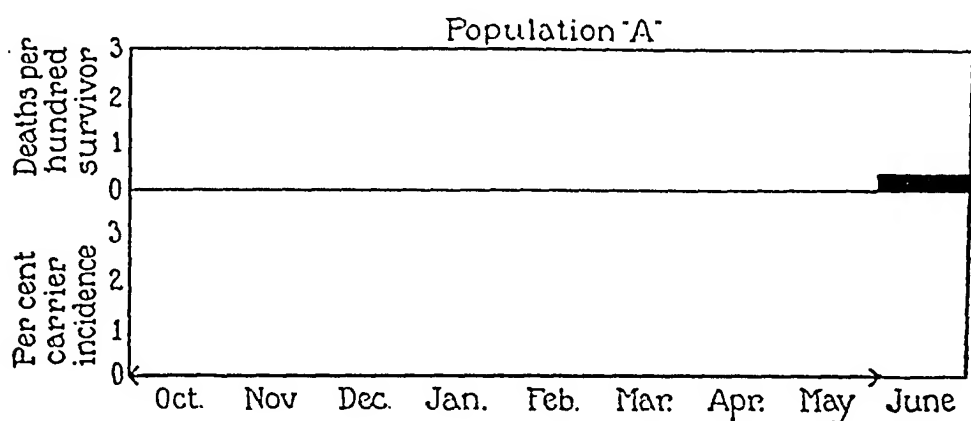
Date	Number examined	Number of carriers	Per cent carriers
12/21/27	196	0	0.0
1/ 5	183	0	0.0
1/18/28	117	0	0.0
2/29	98	0	0.0
3/28	76	1	1.3
4/18	50	10	20
4/25	118	19	16
5/23	119	21	18.5

test, a new house with three compartments was constructed and the entire plant thoroughly cleaned and disinfected. As a result, there occurred considerable shifting of groups. For the purpose of this discussion, however, the entire population can be considered as composed of three distinct divisions, A, B, and C.

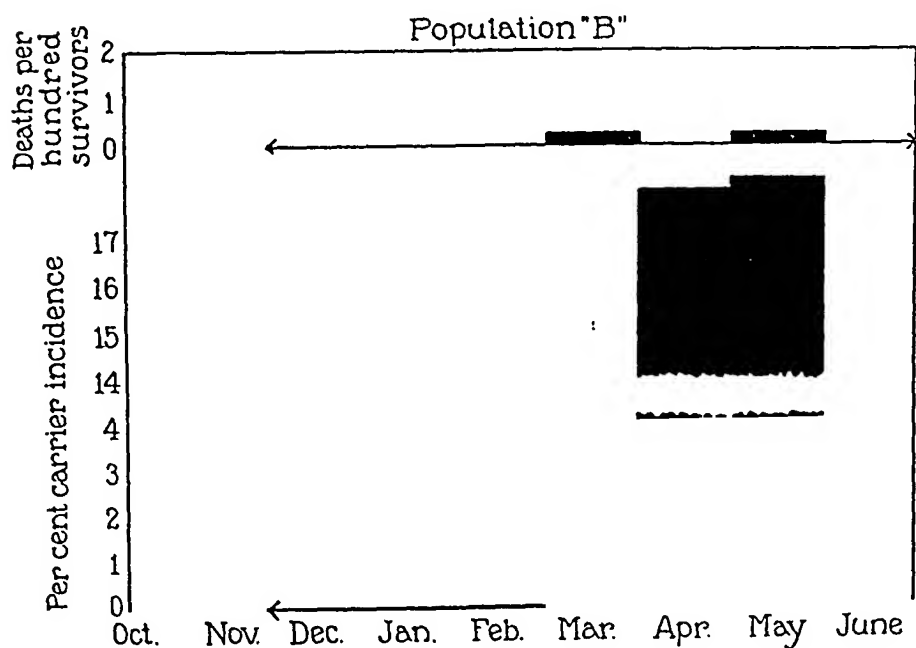
*Population A.* Consisted of 396 pullets, offspring of hens and cockerels of



House III of the previous year. These birds were born and raised in confinement and hence were supposed to have had no contact with other birds. They were kept in Houses I and II. A representative sample was examined at monthly



TEXT-FIGURE 4



TEXT-FIGURE 5

intervals from November, 1927 to May, 1928 inclusive. No carriers of *P. avicida* were detected (Table III). During this period, one bird died of fowl cholera (Table VI, Text-Figure 4).

TABLE V

*Results of Carrier Tests; Population C, Belle Meade, N. J., 1927-1928*

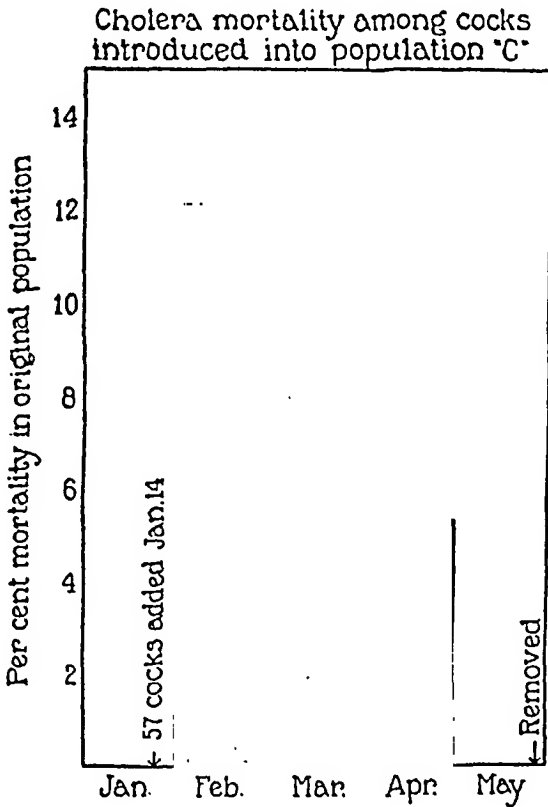
Date	Number examined	Number of carriers	Per cent carriers	Average
10/20/27	95	7	7.4	7.3
10/26	138	10	7.2	
11/ 2	200	23	11.5	4.5
11/16	142	3	2.1	
11/18	74	1	1.3	
11/30	214	7	3.2	
12/ 7	160	5	3.1	2.5
12/14	197	4	2.0	
2/ 1/28	159	4	2.5	2.8
2/ 7	314	11	3.5	
2/16	240	6	2.5	
3/ 7	227	8	3.5	5.1
3/14	207	21	9.9	
3/21	117	5	4.3	
3/28	107	3	2.8	
4/ 4	89	1	1.1	0.7
4/11	106	0	0.0	
4/18	92	1	1.1	
5/16	122	4		3.2

TABLE VI

*Mortality—Belle Meade, N. J., 1927-1928*

Month	Cholera				Non-cholera	Total
	Pop. A (396)	Pop. B (714)	Pop. C (653)	Unknown		
October	0	0	0	0	0	0
November	0	0	4	0	6	10
December	0	0	2	1	44	47
January	0	0	1	0	23	24
February	0	0	3 Females 7 Males	0	17	27
March	0	1	9 Females 8 Males	0	10	28
April	0	0	0 Females 3 Males	1 Female 1 Male	24	29
May	0	1	3	0	18	22
June	1	0	0	0	3	4

*Population B.* Consisted of 714 pullets similar to those in Population A, save that they were allowed to run about the farm at will until December, 1927. They were then placed in Houses III and IV. A sample lot was tested each month for carriers (Table IV). None was found until March, 1928. At that point, 1.3 per cent of the sample of 76 proved positive; in April 18 per cent of 168 birds tested, and in May 18.5 per cent of 119 so examined. In spite of this, only two deaths from cholera occurred during the year, one in March and one in May (Table VI, Text-Figure 5).



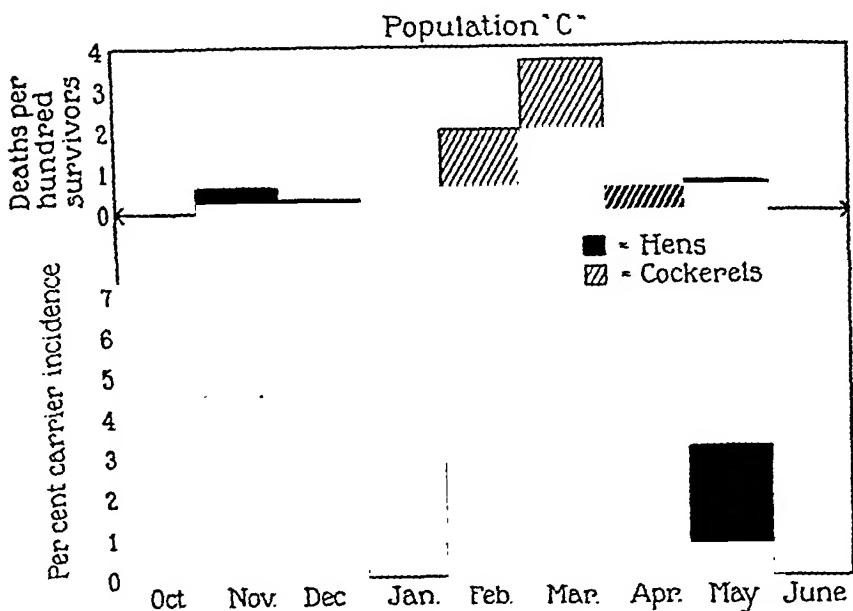
TEXT-FIGURE 6

*Population C.* Comprised the breeding hens selected on the basis of high egg production from the pullets of Houses I, II, and IV. Fifty-seven cockerels, previously shown to have been free of *P. avicida*, were added in January, 1928. The effort to remove all carriers from this group was continued, but at best, seven days elapsed between test and elimination.

Tests for carriers were made from October, 1927 to May, 1928. Each bird had received from two to four examinations. The results, given in Table V, indicate that the population was never free of carriers, in spite of the removal of positive

cases after each test. Nevertheless, the rate was low, and declined progressively as the number of tests increased, until March, 1928, when, coincident with the rise in number of carriers in Population B, an increase in carrier incidence occurred. Subsequently, in April and May, it declined again.

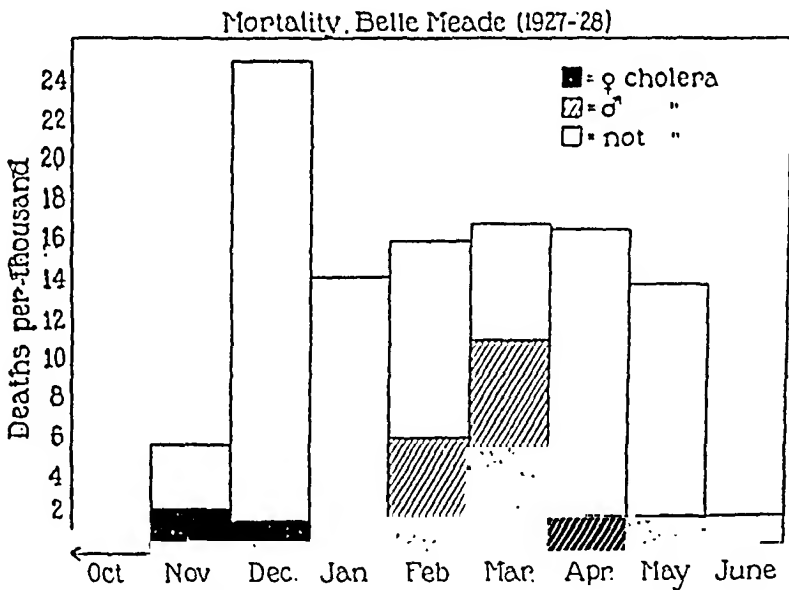
The mortality in Population C is given in Table VI. Four cholera deaths occurred in November, two in December, and one in January. In February three hens and seven cockerels died of cholera, in March nine hens and eight cocks, April three cocks, and May a total of three (Text-Figures 6 and 7).



TEXT-FIGURE 7

This series of observations during 1927-1928, together with those made during the previous year, suggests further conclusions as to the mode of spread of endemic fowl cholera. In the first place, it appears probable that the infection survived over the summer in "healthy" carrier pullets and hens and was spread by them during the following winter throughout the new breeding stock. Thus, the breeding stock C was known to be infected at the outset, whereas the pullets, A and B, were not. Spread took place among the breeders, C, then to some of the pullets, B, presumably because of some technical error. However, no infection took place among the well-protected group of pul-

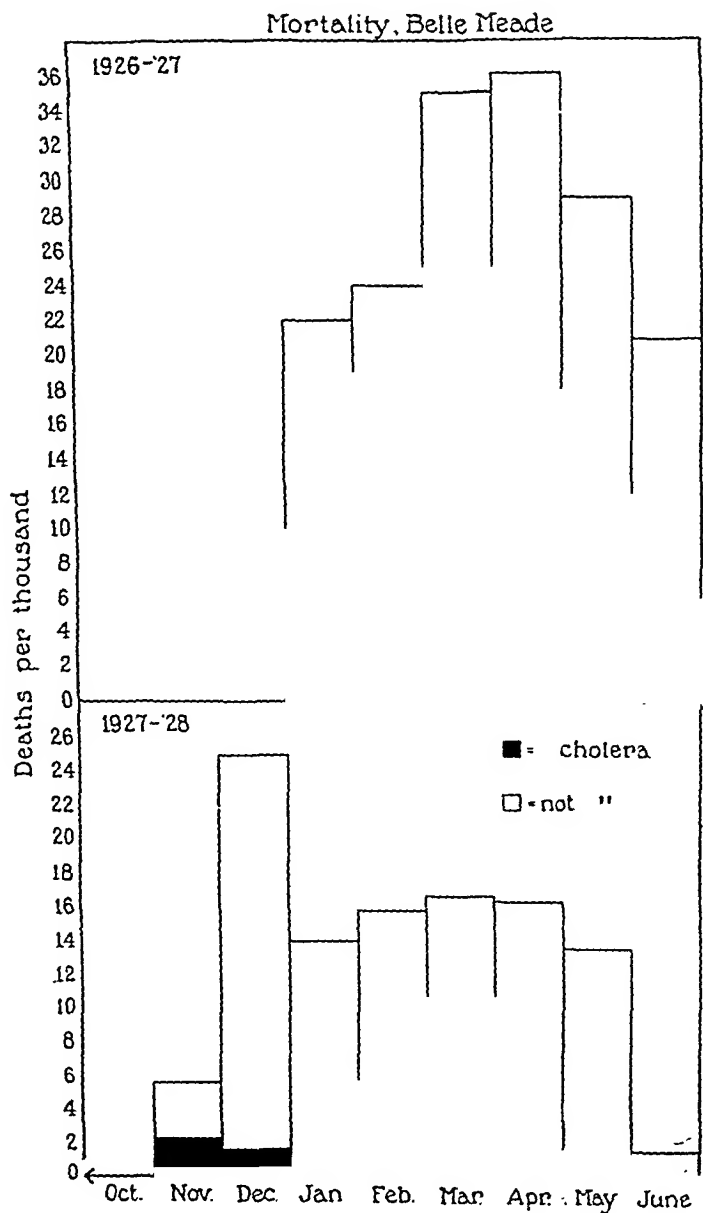
lets, A. In the next place, it seems that the infection may assume the form of an epidemic when susceptible immigrants are brought into the community. This was noted when cocks were added to the group of hens designated Population C. Further evidence was forthcoming to indicate the determining rôle of host resistance in the spread of infection in that Population B, although exposed to a considerable risk, did not suffer an epidemic. The sudden increase in the number of carriers in Population B during March and April, without an accompanying rise in number of fatalities, can be explained at present only



TEXT-FIGURE 8

by assuming that the resistance of the community was enhanced by the seasonal influences of spring.

Three additional points remain to be considered. First, the amount of *P. avicida* infection in this community was seen to vary according to season in 1927-1928 as in 1926-1927. During both years, the amount of carrier infection and fatal infection reached its maximum in winter and minimum in summer (Text-Figure 9). Second, the number of cholera deaths was observed usually to vary directly with the number of *P. avicida* carriers. In Population A, for example, there were no cases of carrier infection and no fatal cholera cases.



TEXT-FIGURE 9

In Population C, carrier rate and cholera mortality rate varied together. Both decreased progressively from October to January and then increased during February and March. In Population B, however, this relationship was marked. Presumably the spring increase in resistance of the fowl limited the infection to the carrier state.

The third point to be discussed is the effect of removing *P. avicida* carriers from Population C. Unfortunately, technical and practical difficulties prevented the completion of a critical test with suitable control. It may be noted, however, that in spite of the fact that severe outbreaks of cholera had occurred each winter for the past several years, no such epidemic occurred in 1927-1928. Indeed, the cholera mortality was less than one-fifth that of the previous year (Text-Figure 9). The carrier rates as well show this marked decrease. In 1927-1928, cholera was confined almost entirely to the breeding Population C, and in this community a comparison of carrier rate and mortality rate figures for the years 1926-1927 and 1927-1928 demonstrates the marked limitation of the infection. There is some reason, therefore, to believe that the spread of fowl cholera may be controlled by the elimination of carriers.

### *Bacteriology*

Strains of *P. avicida* recovered from the Belle Meade flock during 1926-1927 and 1927-1928 were studied and compared. The results of these tests are as follows: the 1926-1927 strains were apparently all of the "blue" colony form (1a), the 1927-1928 strains "blue," except for an occasional culture with the narrow acid agglutination zone and low antiserum agglutination titre characteristic of the "intermediate" colony form (1a).

The acid agglutination zone of thirty-three 1926-1927 strains and thirty-one 1927-1928 strains was determined. The results are given in Table VII. The 1926-1927 strains, with one exception, showed the characteristic "blue" colony type of reaction, agglutination from pH 2.4 to 5.4, while four of the 1927-1928 strains reacted only in the more acid mixtures. No distribution of cultures on the basis of time, or place of isolation was evident. Carrier and autopsy strains behaved in a similar manner.

All strains were tested in "Pa" serum diluted 1:100 (1a). All

1926-1927 cultures were agglutinated strongly; eleven obtained in 1927-1928 failed to agglutinate. Beside this they showed a narrow

TABLE VII

*Zones of Acid Agglutination of Strains of P. avicida Obtained from Carriers and Cases of Fowl Cholera at Belle Meade, N. J.*

Agglutination zone	Number of strains reacting	
	1926-1927	1927-1928
None	0	4
pH 2.4-3.2	1	1
2.4-3.5	0	1
2.4-4.0	0	1
2.4-4.2	0	1
2.4-5.0	0	6
2.4-5.3	0	4
2.4-5.4	21	0
2.5-3.5	1	0
2.5-5.0	1	0
3.0-3.5	1	0
3.0-4.0	2	0
3.0-4.2	1	6
3.0-5.0	1	4
3.0-5.4	3	1
3.0-5.9	0	2
3.2-3.5	1	0
Total number.....	33	31

TABLE VIII

*Agglutination Titre in "Pa" Antiserum of Strains of P. avicida Obtained from Carriers and Cases of Fowl Cholera at Belle Meade, N. J.*

Serum dilution titre	Number of strains agglutinated	
	1926-1927	1927-1928
1:250	—	7
1:500	3	15
1:1000	9	43
1:2000	7	2
1:4000	7	—

zone of reactivity in acid buffer. The titre of twenty-six 1926-1927 strains and sixty-seven 1927-1928 strains was tested in "Pa" serum



(Table VIII). Of the cultures obtained the first year, three agglutinated to a dilution of 1:500, nine to 1:1000, seven to 1:2000, and

TABLE IX  
*Virulence of Carrier and Autopsy Strains, 1927-1928*

Strain	Source	Isolated	Tested	Per cent dead
695	Autopsy	2/ 3/28	2/ 8/28	0
696	"	1/ 3/28	2/ 8/28	0
714	Carrier	2/16/28	2/20/28	0
715	"	2/16/28	2/20/28	0
717	"	2/16/28	2/20/28	0
718	"	2/16/28	2/20/28	0
728	Autopsy	3/ 7/28	3/13/28	10
729	"	3/ 7/28	3/13/28	0
730	"	3/ 7/28	3/13/28	0
733	"	3/ 7/28	3/13/28	0
745	Carrier	3/14/28	3/21/28	0
746	"	3/14/28	3/21/28	0
747	"	3/14/28	3/21/28	0
748	"	3/14/28	3/21/28	0
749	"	3/14/28	3/21/28	0
750	"	3/14/28	3/21/28	0
751	"	3/14/28	3/21/28	0
752	"	3/14/28	3/21/28	0
770	"	3/14/28	3/28/28	10
779	Autopsy	3/14/28	3/28/28	0
781	"	3/14/28	3/28/28	20
783	Carrier	3/28/28	3/28/28	0
784	Autopsy	3/14/28	3/28/28	20
785	"	3/14/28	3/28/28	10
792	"	3/29/28	4/ 5/28	0
793	"	3/29/28	4/ 5/28	0
794	Carrier	3/28/28	4/18/28, 4/25/28	0
795	"	3/28/28	4/18/28, 4/25/28	0
796	Autopsy	3/28/28	4/26/28	0
798	"	3/29/28	4/19/28, 4/26/28	0
801	Carrier	4/18/28	5/ 2/28	0
805	"	4/18/28	5/ 2/28	0
813	Autopsy	4/18/28	5/ 2/28	0

Average mortality: Carrier strains..... 0.6%  
Autopsy strains..... 4.0%

seven to 1:4000, while of those received the second year, seven agglutinated in a dilution of 1:250, fifteen to 1:500, forty-three to

1:1000 and two to 1:2000. Three additional sera were prepared for 1927-1928 strains. Two of these strains agglutinated to titre in "Pa" serum; the other, not at all. The three antisera made from these cultures agglutinated the "Pa" strain, and their specific agglutinins were absorbed by "Pa" antigen. Conversely, "Pa" agglutinins were absorbed from the "Pa" serum by the three 1927-1928 antigens. Hence, no serological differences in these strains could be proved.

The virulence of a number of 1926-1927 strains was tested by the method previously described (1a). None of them killed. The following year, twenty carrier strains and sixteen autopsy strains from Population C were tested. A very low mortality resulted (Table IX). The titrations indicate that the virulence of all strains was similar; 1926-1927 strains were similar in this respect to 1927-1928 cultures, "carrier" strains similar to "autopsy" strains, and strains from houses with relatively little cholera the same as those from populations where cholera was most frequent.

#### SUMMARY

An investigation of endemic fowl cholera, the common form of the disease in this locality, has been made at a poultry farm in Belle Meade, N. J. The focus or reservoir of *P. avicida* proved to be the healthy pullets which had become carriers the previous year and which were selected as the breeding stock for the ensuing season. From these carriers, the organisms spread and gave rise during the winter months to the various forms of infection, including the carrier state, localized upper respiratory disease, and typical cholera. Strains of *P. avicida* were in general very similar and of the "blue" colony type. They were all of the same low degree of virulence; no differences were demonstrable between a) "carrier" and "autopsy" strains, b) strains where the infection was spreading and severe, and strains where disease was rare, and c) "autumn" and "winter" strains. In general, a relatively high carrier rate was accompanied by a high mortality rate, although in one instance, a community with high carrier incidence plus a probable high degree of host resistance suffered but little fatal infection. An attempt was made to reduce the amount of cholera by the removal of carriers. The results of this measure indicate that such a procedure is both effective and practical.

The authors desire to express thanks to Mr. C. B. Hudson for his valuable assistance in this work, and to Mr. Max Bogner, the owner of the Belle Meade Farm for his cooperation and aid.

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# EARLY PULMONARY LESIONS IN PARTIALLY IMMUNE ALCOHOLIZED MICE FOLLOWING INHALATION OF VIRULENT PNEUMOCOCCI

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In preceding papers (1) it has been shown that virulent pneumococci generally disappear from the lungs of normal mice within a few hours after inhalation and do not give rise to generalized infection. In alcohol-intoxicated mice, however, pneumococci persist in the lungs for a longer period and a fatal septicemia frequently follows. It has further been shown (2) that a high degree of immunity is produced in mice following repeated inhalations of live pneumococci and that a less marked immunity results from exposure to a spray of dead organisms. In the instances in which non-immune mice succumb as the result of infection following a pneumococcus spraying while alcoholized, death is due to general septicemia and there is no evidence of localized infection in the lung. However, mice which have been rendered partially immune by inhalation of live or killed pneumococci and are later alcoholized and exposed to virulent pneumococci show definite evidence of localized pulmonary infection.

In the foregoing experiments only animals which had succumbed to the pneumococcus infection were studied. Furthermore, the entire lung was not examined. There was the possibility that:—(1) some mice might have developed pneumonia but subsequently recovered, and (2) that early pneumonic lesions might have been missed as all the lobes were not examined histologically. In order to accurately determine the incidence of pulmonary localisation and to detect the initial pulmonary reactions in partially immunized mice following inhalation of virulent pneumococci while alcoholized, the following experiments were undertaken. This paper is a report on the examination of serial sections of the lungs of 81 partially immunised mice which

were allowed to inspire virulent homologous pneumococci while alcoholized.

### *Method*

The mice to be immunized were placed in the spray chamber previously described and sprayed at intervals of 2 to 3 days with broth cultures of pneumococci which had been killed by heating to 60° for 30 minutes, or heat killed pneumococci suspended in salt solution.

Ten days after the last spraying the mice were alcoholized by intraperitoneal injection of 1.5 c.c. of a 10% solution of alcohol in saline. 1 hour after the administration of the alcohol, the animals were sprayed with 50 c.c. of virulent live pneumococcus culture and allowed to remain in the spray box for 1 hour. At intervals of 6, 12, 18 and 24 hours following spraying, groups of at least 9 mice were killed by chloroform.

After the heart's blood was cultured in broth, the lungs were removed in toto and dropped into Zenker's fluid to which 5% acetic acid had been added. They were paraffin embedded and serial sections were cut 12 microns thick. Every 12th section was examined as routine, and where lesions were discovered the intervening sections were examined.

The pulmonary lesion called chronic consolidation occurs in about 3 per cent of apparently healthy stock mice. Both in the gross and microscopically the lesions are readily differentiated from lesions due to the pneumococcus.

### EXPERIMENTAL

*Group I.* 44 mice which had been partially immunised by spraying with whole killed cultures of pneumococci were exposed to inhalations of virulent type I pneumococci while alcoholized. The incidence of pulmonary lesions and the results of the heart's blood culture of these mice are given in Table I.

From this table it is seen that of the 10 mice killed in 6 hours, the lungs were normal in 4, and congested or edematous in 5. In only one animal was there any evidence of localisation of the infection as indicated by an inflammation in the alveolar wall.

The lungs of 7 of the 10 mice sacrificed in 12 hours appeared normal; in one chronic consolidation was present, in one congestion and edema, and in one the alveolar walls were inflamed.

The lungs of 6 of the 10 mice killed in 18 hours were normal; in one chronic consolidation and in three congestion were present.

The lungs of 10 of the 14 mice killed in 24 hours were normal; in 2 chronic consolidation occurred and in 2 congestion and edema.

In none of the mice killed in 6 hours was the heart's blood culture positive and the pneumococcus was recovered from only one and two mice of those killed in 12 and 18 hours respectively. 7 or 50% of the mice killed at 24 hours, however, showed positive heart's blood cultures.

*Group II.* 37 mice previously partially immunised by exposure to heat killed pneumococci were intoxicated and sprayed with virulent homologous pneumococcus. The results of the blood cultures and histological examination of the lungs of these mice are given in Table II.

TABLE I

*Pulmonary Lesions in Lungs of Mice Partially Immunised by Inhalations of Heat-Killed Whole Cultures of Pneumococcus Type I*

Time killed after inhalation of virulent Type I pneumococcus while alcoholised											
6 hours			12 hours			18 hours			24 hours		
No.	Blood culture	Pathology	No.	Blood culture	Pathology	No.	Blood culture	Pathology	No.	Blood culture	Pathology
A1	—	C	B1	—	N	C1	—	N	D1	+	C.C.
A2	—	C.E.	B2	—	Early I.I.	C2	+	C	D2	+	C.C.
A3	—	H.E., C	B3	—	C.C.	C3	—	N	D3	—	N
A4	—	Early I.I.	B4	+	C., H.E.	C4	—	N	D4	—	N
A5	—	N	B5	—	N	C5	—	N	D5	+	N
A6	—	N	B6	—	N	C6	—	N	D6	—	N
A7	—	C	B7	—	N	C7	—	C	D7	—	N
A8	—	N	B8	—	N	C8	—	C	D8	—	N
A9	—	C.E.	B9	—	N	C9	+	C.C.	D9	—	N
A10	—	N	B10	—	N	C10	—	N	D10	+	N.
									D11	+	C
									D12	+	C.E.
									D13	—	N
									D14	+	N

N = normal.

C = congestion.

E = edema.

H.E. = Hemorrhagic edema.

I.I. = Interstitial inflammation i.e. lesion limited to alveolar wall.

R = Red hepatisation.

C.C. = Chronic Consolidation.

— = negative blood culture.

+

The lungs of 6 of the 9 mice killed in 6 hours appeared normal; in 2 they were congested and edematous and in one animal there was evidence of an attempt at localisation in the form of an inflammation in the alveolar wall.

The lungs of 8 of the 9 mice chloroformed at 12 hours were normal; in one they were congested and edematous and in one chronic consolidation was present. Localised areas of red hepatisation were found in 2 of the remaining 3 animals and interstitial inflammation was present in one.

Normal lungs were found in 5 of the 9 mice sacrificed at the end of 24 hours. Chronic consolidation occurred in 2 mice, congestion and edema in one, and red hepatisation in one.

The cultures of the heart's blood of all the mice killed after 6 and 12 hours remained sterile. Positive heart's blood cultures were obtained from 2 of the animals killed after 18 hours, while the blood of 5 or 55% of the mice sacrificed after 24 hours contained pneumococci.

TABLE II

*Pulmonary Lesions in Lungs of Mice Partially Immunised by Inhalations of Heat-Killed Pneumococcus Type I Vaccine*

Time killed after inhalation of virulent type I pneumococcus while intoxicated											
6 hours			12 hours			18 hours			24 hours		
No.	Blood culture	Pathology	No.	Blood culture	Pathology	No.	Blood culture	Pathology	No.	Blood culture	Pathology
A1	—	N	B1	—	N	C1	—	N	D1	—	C.C.
A2	—	N	B2	—	C.C.	C2	—	N	D2	—	N
A3	—	N	B3	—	N	C3	+	I.I.	D3	—	N
A4	—	N	B4	—	N	C4	—	Local R	D4	—	C.E.
A5	—	I.I.	B5	—	N	C5	—	N	D5	+	R
A6	—	N	B6	—	N	C6	—	C.C.	D6	+	N
A7	—	C.E.	B7	—	N	C7	—	N	D7	+	N
A8	—	N	B8	—	N	C8	—	N	D8	+	C.C.
A9	—	C.E.	B9	—	N	C9	+	Local R	D9	+	N
						C10	—	C.E.			

N = normal. I.I. = interstitial inflammation i.e. lesion limited to the

C = congestion. alveolar wall.

E = edema. R = red hepatisation.

— = negative blood culture.

+

On gross examination the lungs of these 81 mice showed, in a few instances, congestion and chronic consolidation. Of these, 8 must be excluded because of the presence of chronic consolidation which would mask any slight lesions that might have been present. The lungs of 51 or 71% of the remaining mice were normal, and congestion and edema occurred in 15 or 21%. Evidence of pulmonary localisation occurred in 7 or 9%. The inflammatory process was limited to the alveolar walls in 2 mice killed after 6 hours, and in 2 animals killed after 18 and 24 hours respectively. In 3 mice exudation into the alveoli was the predominant feature and the lesions were classed as red hepatisation. Small areas of beginning con-

solidation occurred in 2 mice killed after 18 hours and the greater portion of one lobe was involved in a mouse killed in 24 hours.

Of the 81 mice, the pneumococcus was recovered from the heart's blood in 17 or 22%. Only one of the mice killed within 12 hours had a positive blood (5%), while 4 of those killed after 18 hours (20%) and 12 of the animals sacrificed after 24 hours showed a septicemia (52%). Of the 17 positive blood cultures, 4 occurred in mice with chronic consolidation and therefore must be excluded. Of the remaining 13, 6 occurred in mice with normal lungs, 4 were associated with congestion and edema, and 3 with inflammatory lesions.

### *Microscopic Pathology of the Early Pneumonic Lesions*

This study is limited to serial sections of the 6 lungs mentioned above which showed inflammatory lesions not of the spontaneous chronic type often found in mice.

The nature of the early lesion is exemplified by the reaction occurring in the lungs of a mouse killed 6 hours after spraying:

An exudative inflammatory process involves 2 lobes, the caudal and ventral. The lesion is 2-4 m.m. wide, and does not reach the hilum nor the pleura. The inflammation is limited to the alveolar wall. The only exudation into the lumen has occurred at the junction of atria and bronchioli. Neither the large, nor small vessels nor the pleura are involved. In sections of this thickness (12 m.m.) it is not possible to say whether the thickening of the alveolar wall, with the accompanying exudate which is particularly rich in polymorphonuclear leucocytes, is due to inflammatory cells within or without the capillaries or to a combination of both. An area of atelectasis occupying about one fifth of the whole lobe is situated around the inflammation but the surrounding pulmonary tissue is congested. The area of inflammation appears to be continuous. The bronchial lymph nodes are not involved and no lymphatic involvement is apparent.

After 18 hours 2 lungs showed exudation into the alveolar lumen sufficiently marked to be considered the predominant lesion and were classed as stages of beginning red hepatisation.

### DISCUSSION

The earlier studies on the production in the lower animals of lobar pneumonia with the pneumococcus were concerned at first with establishing the etiological rôle of this micro-organism.

The methods employed were direct injection of lung exudates into the thoracic cavity, trachea, peritoneum, and blood.

To Thalamon (3) is given the credit for first producing pulmonary lesions in rabbits by the intrathoracic method. Gameleia (4) carefully worked out the



relative resistance or susceptibility of the various lower animals to intraperitoneal injections of pneumococcus. He found that white mice and rabbits are most susceptible to pneumococcus infection while guinea pigs, dogs, sheep and man are increasingly more resistant. He produced pneumonia by intrathoracic inoculation in rabbits, dogs, and sheep. Gamaleia further showed that, since virulent strains would rapidly kill susceptible animals with an overwhelming septicemia without any pulmonary localisation, in order to produce pulmonary lesion in these animals an attenuated strain must be used. Because of this fact later investigators have attempted to determine what factors determine pulmonary localisation of the infection in susceptible animals. Wadsworth (5) expressed the belief that there exists a subtle equilibrium between the resistance of the animal on the one hand and the virulence of the organism on the other. He found that normal rabbits died of a fulminating septicemia without pulmonary lesions following intra-tracheal injections of highly virulent pneumococci. But on the other hand he could cause pneumonia to develop in normal animals by the use of less virulent cultures or by the use of virulent cultures in animals which had acquired a certain degree of immunity. In dogs, less susceptible animals, which were used by Lamar and Meltzer (6) and Wollstein and Meltzer (7), pneumonia was constantly induced after intra-bronchial insufflation of large amounts of culture. More recent work by Blake and Cecil (8) on monkeys has emphasised the bronchiogenic origin of the disease and these observers state that "lobar-pneumonia has been consistently produced in normal monkeys by the intra-tracheal injection of minute amounts of pneumococcus culture." Winternitz and Herschfelder (9), however, working on rabbits had previously found that it was necessary to introduce the organisms deep into the lower respiratory tract to produce pneumonia and Winternitz (10), considers that the lymphatic origin of the disease cannot be eliminated, in spite of the experiments quoted above, on the ground that by the method of intra-tracheal inoculation it is impossible not to infect the peribronchial tissue as the needle is being withdrawn.

It will be readily noted that all the "successful results" reported, without discriminating between those of doubtful and actual value, have been by intra-thoracic or intra-tracheal inoculation, there being no experimental proof of the haematogenous origin of the disease. But even the success of these methods has not been universally accepted, as many observers (Welch & Canfield (11), Krause and Pansini (12)) failed to confirm Gameleia's results, and succeeded only in producing lesions which they did not consider comparable to the typical lobar pneumonia as seen in man.

The present method, that of inhalation, is generally admitted to have failed, although some investigators believed that an occasional positive result was obtained. Selter (13) was unable to produce pneu-

monia in rabbits and guinea pigs exposed in a box to a spray of pneumococci which had been isolated from an epidemic of pneumonia in these animals. The inhalation method was employed in the present experiments because it is less artificial than the others and removes the objection of mechanical injury.

The points which must be considered in any discussion of the pathogenesis of pneumonia are: 1—the route by which the causative agent reaches the lung and 2—the site of initial invasion and spread of the infection in the pulmonary tissue.

### *Mode of Invasion*

The infecting organism may reach the lungs by the blood stream, lymphatics, or directly from the respiratory tract.

The lymphatic route presupposes that entrance takes place in the bronchi or upper respiratory tract and that the organisms progress along the lymphatics to the lymph nodes at the root of the lung. The lymphatics become blocked by the inflammatory reaction set up by the organisms causing a back pressure and the infection is first noticed in the lung tissue. Or the organisms may pass through the lymph nodes into the thoracic duct and reach the lung by the pulmonary artery. In the mouse the lung is scant in lymphatics and we have seen no evidence in our sections of any such marked lymphatic involvement as this mode of infection would presuppose. It is certain that the lymph nodes themselves are not early affected.

The occurrence of pulmonary changes in the presence of negative blood cultures in the first 6 hours would at first sight appear to favor theory of direct primary invasion of the pulmonary tissue itself, particularly as the earliest lesions encountered have always been in the alveolar wall. But these pathological findings may be equally well explained on the ground that the infecting agent reaches the alveolar wall by the capillaries and is localised there until later when a secondary septicemia occurs.

The adherents of the hematogenous method of infection assume that the pneumococcus gains entrance at some point in the respiratory tract and is carried either directly into or through the lymphatics into the blood stream to the lungs where the microorganisms set up a

pneumonia. A selective filtration action on the part of the lungs is assumed to take place. No direct experimental evidence has yet been brought forward to prove this hypothesis. Attempts to test its validity are now in progress. The fact that pneumococci may be recovered from the liver, spleen and kidney of rabbits and guinea pigs following inhalation of pneumococci, though the blood culture is negative, indicates that a transient pneumococcus septicemia may occur. It has further been shown by daily blood cultures following spraying that a transient pneumococcus septicemia occurs frequently in rabbits. There probably are few organisms free at any one time in the circulating blood and furthermore these few bacteria may be within leucocytes, not multiplying in the blood. The occasional organisms which reach the blood are probably rapidly filtered out by the tissues and locally destroyed.

The failure to obtain positive blood cultures in the 2 mice which showed evidence of pulmonary lesions may be due to the circumstance that re-invasion of the blood stream from these foci of infection had not taken place after, possibly, transient primary septicemia. 2 of the 4 mice killed after 18 hours, however, with lungs showing lesions, had a generalised septicemia.

#### *Nature of the Initial Lesion*

In a proportion of partially immunised and intoxicated mice, infection after inhalation of virulent pneumococci is localised in the lungs. In these mice the initial lesion is an inflammation of the alveolar wall (extra or intra vascular). Blake and Cecil incline to the belief that the early lesion is in the interstitial tissue, while Pernar (14) speaks of the disease as an exudative alveolitis throughout. Since cellular exudation into the alveoli occurs very early, the nature of the initial lesion can only be determined in mice killed soon after infection. But these early lesions are so minute that there is no gross pathological change except perhaps congestion, the significance of which is debatable in sacrificed animals. The only safe method is to cut serial sections of the whole lung in order to avoid missing any minute early lesions.

In the present study localisation of the pneumococcus infection was present in the lungs of 7 of 71 mice. Although this is too small a

number of animals from which to draw definite conclusions, the outcome agrees with that of previous experiments in which the observations were made on mice which had succumbed to the infection. The lymphatic spread from the original focus as observed in monkeys by Blake & Cecil certainly does not occur in the mouse. The infection in this species is progressive and is localized to the alveolar wall. In previous experiments pleurisy occurred when extensive lymphatic involvement was found and the animals died before a well defined localisation had time to develop. We believe that experimental pneumococcus pneumonia, at least in the mouse, begins in the alveolar wall, possibly within the capillaries.

#### SUMMARY

1. The serial section examination of 71 partially immune alcoholised mice which were killed at intervals following the inhalation of virulent pneumococci showed pulmonary localisation in 7 or 9%.

2. In the case of the mouse the initial lesion of pneumococcus pneumonia is in the alveolar wall and the exudate into the alveolar lumen occurs secondarily.

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## STUDIES ON INFLAMMATION

### II. A MEASURE OF THE PERMEABILITY OF CAPILLARIES IN AN INFLAMED AREA

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The accumulation of vital dyes in areas of inflammation has been demonstrated by several investigators. MacCurdy and Evans (1) pointed out that the normal brain and cord always remain free from dye injected intravenously but that areas of damage, such as softening or inflammation, become deeply stained. Bowman, Winternitz, and Evans (2) found that trypan blue injected intravenously stains tubercles in experimental tuberculosis. Subsequently Winternitz and Hirschfelder (3) demonstrated that this dye when injected in experimental lobar pneumonia stains the consolidated area of lung selectively: "The intravenous injection of trypan blue and trypan red gave rise to the usual diffuse staining as described by Bouffard, Goldman, etc., but in addition to this the diseased area of lung showed a much more intense staining than any of the other tissues, while the normal lung tissue was practically normal in color." Lewis (4) found that if the cornea of a rabbit is inoculated with a living culture of the tubercle bacillus, a progressive lesion results characterized by an intense congestion of the conjunctiva. If the animal receives an intravenous injection of trypan red 24 hours or more after such inoculation, the fluid in the anterior chamber of the inoculated eye always becomes colored. Precisely similar results were obtained when abrin was administered in the conjunctiva as an inflammatory irritant. A few years ago McClellan and Goodpasture (5) showed that trypan blue accumulates in lesions of herpetic encephalitis in the rabbit's brain, the injured areas presenting a striking color against the quite unstained healthy brain tissue. Siengalewicz (6) pointed out that general damage to the nervous tissue, such as poisoning with carbon monoxide or with salvarsan, is followed by marked staining of the damaged areas by trypan blue. Ramsdell (7) injected trypan blue into the veins of rabbits and guinea pigs previously treated with foreign serum and found that injection of the same serum into the skin of the ear immediately caused local infiltration of the dye into the adjacent tissue. She regarded the infiltration of the dye as an indicator of edematous changes resulting from toxic injury to the capillary endothelium.

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Okuneff (8) found that a thermal irritant favors the passage of vital stains from the blood stream into the area heated. Kusnetzowsky (9) also observed that the local application to the skin of an irritant such as heat or mustard oil causes an accumulation of trypan blue in the inflamed area when the dye has previously been injected into the blood stream. It has recently been demonstrated by one of us (10) that trypan blue injected into the circulating blood enters the site of inflammation rapidly and is fixed there, so that the tissues are deeply stained. Furthermore trypan blue injected directly into the site of inflammation in the subcutaneous tissue or in the peritoneal cavity is fixed in the inflamed area and fails to reach the regional lymphatic nodes. This work clearly showed that there is not merely a rapid accumulation of the dye from the blood stream into an inflamed area, but also that the dye is held in such an area and is unable to drain away through the regional lymphatics.

The object of the work reported here was an attempt to study quantitatively and directly the rate of change of concentration of trypan blue in the capillaries of an inflamed area. It was thought that such direct studies correlated with the preceding studies on the accumulation of dye from the circulating blood into an inflamed area might give some information concerning the change in capillary permeability with inflammation.

### *Method*

An inflammatory reaction was produced in the peritoneal cavity of frogs (*R. catesbiana* and *R. clamitans*) (11) by the injection of about 2 cc. of either 5% aleuronat in Ringer solution or 4% turpentine in olive oil (12). Twelve to twenty-four hours after the injection of the irritant the brain of the frog was pithed, the spinal cord being kept intact in order to preserve a more adequate circulation (13). The frog was then placed on a frog board of the usual type and the abdominal wall was incised from the region of the pubis to the sternum. A loop of intestine was gently drawn out and the mesentery spread over a glass cover on the center of the board, which in turn was placed on the stage of the microscope. 1.5 cc. of aqueous 1% trypan blue was then injected directly into the ventricle. The dye stained the plasma of the mesenteric capillaries almost immediately. The circulation in most cases remained unimpaired. Gradually, over a period of about 10 minutes or less, most of the dye diffused out of the capillaries.

All these observations were duplicated in about the same number of normal animals as controls. Throughout the experiments particular attention was paid to the character of the peritoneal exudate, the appearance of the mesentery and the activity of the capillary circulation. When the circulation is in partial or complete stasis the capillaries may show variable changes in concentration of the dye, and these changes therefore, were studied only in capillaries in which the circula-

tion was active. Although prolonged exposure of the mesentery in a normal frog is sufficient injury to set up an inflammatory reaction, the duration of each experiment was so short that this factor, as judged by the scant migration of leucocytes in our control series, was almost negligible.

A graded series of standards of trypan blue ranging in concentration from 20 mgm. per 100 cc. of water to pure water was made up in glass capillary tubes measuring about 4 to 5 cm. long and about 1 mm. in diameter. These were glued on white cardboard so as to be conveniently handled by the observer. The determinations were made colorimetrically, the attempt being to match the dye in the plasma as seen through the low power objective with the dye in the set of standards. With only a little practice we found this method of estimating the concentration of dye in the plasma to be without difficulty. The observations were usually checked by two people and the mean taken. To avoid preconceptions, one investigator would often make readings while ignorant as to whether the mesentery under observation was inflamed or normal. Readings were made approximately every minute, the last not more than 7 minutes after the first since after that time the dye in the plasma was so dilute as to make accurate determination of the concentration questionable. Although the dye seen through the microscope in the capillaries was matched against standards in glass capillary tubes, nevertheless, since the same technique was used in estimating changes in concentration in both inflamed and normal areas, it is believed that these colorimetric measurements have yielded comparable and valuable data even though we cannot assume that they measure quantitatively the dye within the capillaries.

## RESULTS

In Table I there are tabulated the results obtained for changes in concentration of trypan blue in the capillaries of the normal mesentery with disappearance of the dye. The results are expressed in units comparable to milligrams of dye per 100 cc. as determined by the standard scale, and the observations range over a period of seven minutes. The averages of the observed values are represented in Chart I.

If the concentration of dye is denoted by  $y$  and the time by  $x$ , and if  $\log y$  for each concentration is plotted against  $x$ , a straight line is obtained. This indicates that the relation between concentration of dye in the capillaries and time follows an exponential curve of the "die-away" type:  $y = be^{-ax}$ , where  $b$  and  $a$  are constants, and  $e$  is the base of the natural system of logarithms equal to 2.71 . . . By the method of least squares the following equation is obtained:

$$y = 7.63 e^{-.24 x} \dots\dots\dots (1)$$



TABLE I

*Changes in Concentration of Trypan Blue in the Capillaries of the Normal Mesentery*

Frog No.	Time in minutes						
	0	1	2	3	4	6	7
	Units comparable to milligrams per 100 cc.						
1	10	4	3	3	2.0	1.6	—
2	10	7	5	3	1.6	1.6	—
3	10	7	7	5	3.0	3.0	2.0
4	8.5	—	4	—	—	—	2.0
5	10	—	—	5	—	1.6	—
6	7	—	—	—	2.0	—	1.3
7	10	—	4	3	—	—	—
8	8.5	—	—	4	—	—	1.06
9	7	5	4	3	3.0	1.5	1.5
Average....	9.00	5.75	4.50	3.71	2.32	1.86	1.57

TABLE II

*Changes in Concentration of Trypan Blue in the Capillaries of the Inflamed Mesentery*

Irritant	Frog No.	Duration of inflammation	Time in minutes						
			0	1	2	3	5	6	7
		hrs.:mins.	Units comparable to milligrams per 100 cc.						
Aleuronat	10	12 : 30	10	4.0	2.8	1.6	1.3	—	1.2
	11	20 : 30	10	—	—	4.0	1.0	0.0	—
	14	15 : 00	10	4.0	1.6	1.06	—	0.73	0.0
	16	18 : 00	10	2.0	—	0.37	—	0.0	—
	18	17 : 30	10	—	1.6	0.0	—	—	—
	19	23 : 00	7	3.0	2.0	1.0	0.7	0.7	0.0
	20	17 : 00	10	5.0	4.0	2.0	1.0	1.0	1.0
Turpentine	12	46	7.8	—	2.8	1.3	0.0	—	—
	13	17 : 30	8.5	3.0	1.06	1.06	—	0.0	—
	15	20 : 00	10	7.0	—	4.0	1.3	0.73	0.0
	17	18 : 00	10	—	—	4.0	—	1.3	0.0
Average.....			9.39	4.00	2.27	1.85	0.88	0.56	0.37

From this equation the calculated values of  $y$  are obtained:

Time ( $x$ ) mins.	Concentration of dye ( $y$ ) Units comparable to milligrams per 100 cc.	
	Observed	Calculated
0	9.00	7.63
1	5.75	6.00
2	4.50	4.72
3	3.71	3.71
4	2.32	2.92
5	—	2.30
6	1.86	1.81
7	1.57	1.42

These values are graphically represented in Chart I. It is seen that there is a fairly close agreement between the calculated and observed figures.

The results obtained for the changes during the first seven minutes in the concentration of trypan blue in the capillaries of the inflamed mesentery are tabulated in Table II. As with the normal values, plotting  $\log y$  against  $x$  points to an exponential type of relationship between the concentration of dye in the plasma and the time (Chart I). The equation obtained by the method of least squares is:

$$y = 6.9 e^{-.42x} \dots\dots\dots (2)$$

The calculated and observed values of  $y$ , graphically shown in Chart I, are as follows:

Time ( $x$ ) mins.	Concentration of dye ( $y$ ) Units comparable to milligrams per 100 cc.	
	Observed	Calculated
0	9.39	6.90
1	4.00	4.53
2	2.27	2.98
3	1.85	1.96
4	—	1.29
5	0.88	0.85
6	0.56	0.56
7	0.37	0.37

Chart I

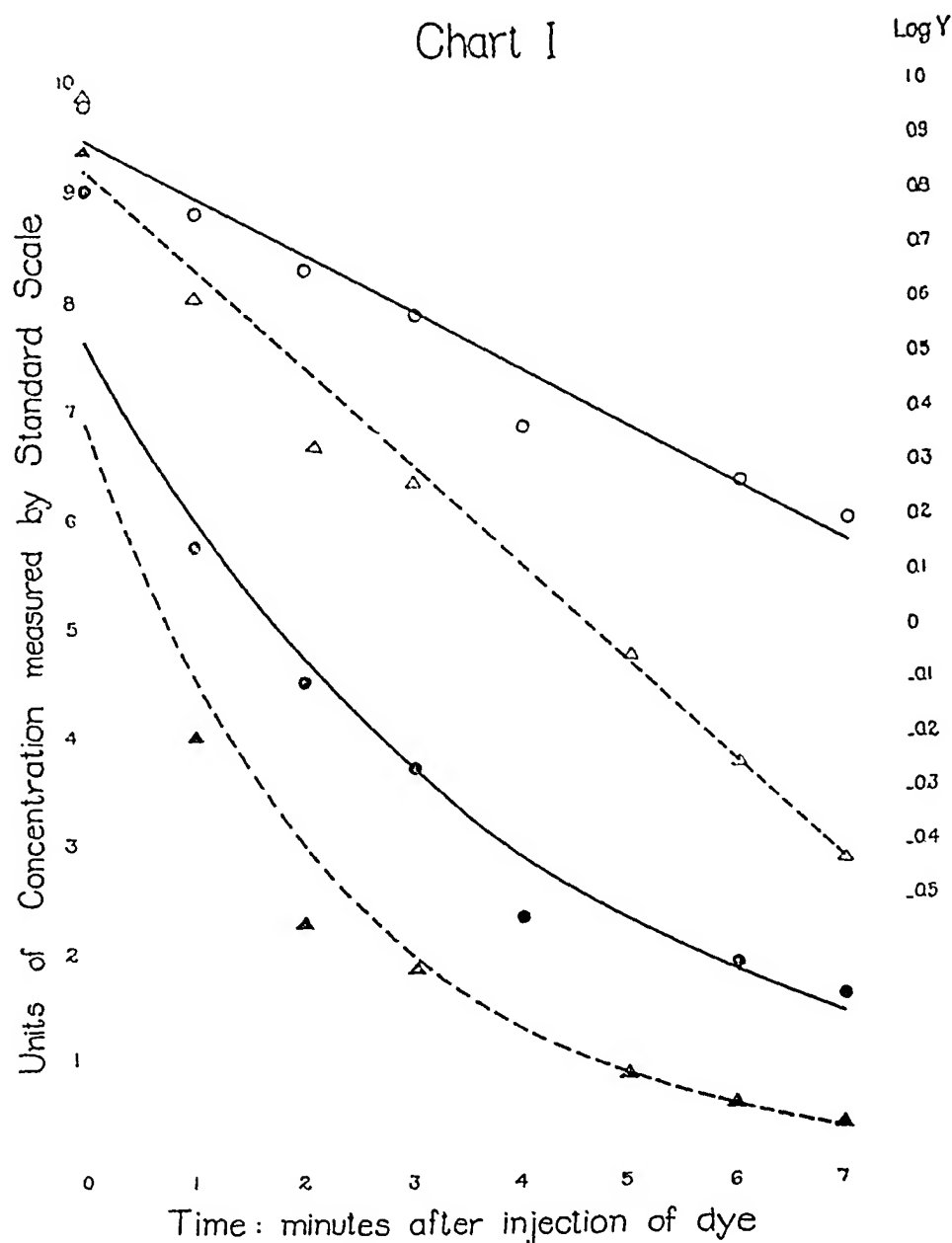


CHART I. The change in concentration of trypan blue in the capillaries of the mesentery. ● observed normal values. ▲ observed values, inflamed mesentery. — concentration of dye in capillaries of normal mesentery (calculated). --- concentration of dye in capillaries of inflamed mesentery (calculated). ○ logarithm of observed normal values. △ logarithm of observed values, inflamed mesentery.

Both equations (1) and (2) appear to be the nearest expressions that describe the observed facts. It is seen that in equation (1)  $a$  equals 0.24, whereas in equation (2) it equals 0.42. Since  $a$  is an index of the slope and consequently of the rate of change of concentration of the dye it is clear therefore that the rate of fall of concentration of trypan blue in the capillaries of an inflamed area is almost twice as rapid as that found in the capillaries of the normal mesentery.

A question now presents itself. Is the rate of change of concentration of the dye in the capillaries an indication of the rate of passage of the dye outward into the extra-capillary spaces? The dye obviously diffuses outward into the extra-capillary spaces, as can be seen by direct observation, and, of course, by the fact that the dye stains the cells of the extra-capillary spaces. This does not necessarily mean that the fall in concentration of dye within the capillaries is a measure of the amount of dye that passes through their walls, for it is conceivable that there may be other factors that change the concentration of dye within the capillaries in an inflamed area. However, in view of previous studies (10) in which it has been shown that trypan blue injected intravenously rapidly passes into an inflamed area, it is believed that the increased rate of fall of concentration is a measure of increased passage of the dye through the capillary wall.

#### DISCUSSION

The above observations show that the rate of fall of concentration of trypan blue in the capillaries after intraventricular injection of the dye is greater in the inflamed than in the normal mesentery. Landis (14) showed by micro-injection studies that the rate of passage of dye solution through the normal capillary wall appears to depend upon the level of capillary pressure, not upon capillary diameter. He demonstrated that the rate of filtration through the capillary wall was directly proportional to the difference between the capillary pressure and the osmotic pressure of the plasma colloids. He also emphasized (13) that the vitality of the capillary wall is of the utmost importance, since injury increases its permeability to proteins. At the same time he demonstrated that the increased filtration through the capillary wall with the use of urethane was due not to a stretched endothelium of the dilated capillary, as Krogh originally believed (15), but rather

to a direct injury of the endothelium by urethane accompanied by a high capillary pressure. Furthermore he showed that capillaries injured by alcohol and mercuric chloride appear to be permeable to the plasma colloids and approximately seven times more permeable to fluids than the normal capillary wall. In view of these facts it seems probable that the increased rate of fall in concentration of trypan blue in the capillaries of the inflamed mesentery might also be caused, as with urethane, alcohol, or mercuric chloride, by a direct toxic effect of the inflammatory irritant on the capillary endothelium, which would thus cause an increase of passage of the dye into the extra-capillary spaces. Such direct injury, causing a fall in the osmotic pressure of the plasma colloids resulting from increased capillary permeability, would adequately account for an increased rate of filtration of the dye in an inflamed area and therefore a greater rate of fall in the concentration of dye within the capillaries.

It is true that the factor of increased capillary pressure cannot be completely ruled out. The work of Hirschfelder (16) would however seem to rule out this factor as being of much significance in the increased filtration of dye in inflammation. This investigator showed that when adrenalin was used to reduce the filtration pressure between the capillaries and the extra-capillary spaces, preventing thus the development of edema, still the inflamed area was stained more deeply than the normal area when trypan blue was injected intravenously. For this reason it is believed that the increased rate of fall of concentration of dye in the capillaries of an inflamed area is primarily due to direct injury of the endothelium by the irritant causing rapid filtration of the dye with increased capillary permeability.

The exponential type of equation which seems to describe adequately the rate of change of concentration of trypan blue in the capillaries brings up an interesting point. This type of curve reaches the abscissa line only asymptotically. It would follow that in these experiments the plasma is never completely free of its dye content. This fact is in accord with the work of Grollman (17) who pointed out that a certain amount of such dyes in the circulation is bound by the plasma protein and therefore does not filter out of the capillaries.

We wish to express our thanks to Dr. Eugene L. Opie and to Dr. M. H. Jacobs for assistance in the preparation of this paper.

## CONCLUSIONS

1. By means of a colorimetric method the concentration of trypan blue in capillaries can be estimated by direct observation and its changes followed as the dye passes out of the circulating blood stream.
2. The change in concentration of trypan blue in the capillaries of both the normal and the inflamed mesentery of frogs can be described by two separate exponential equations of the type:  $y = be^{-ax}$ .
3. From these equations it is found that the rate of fall of concentration following intraventricular injection of the dye is almost twice as great in the capillaries of the inflamed as in those of the normal mesentery. This difference is a measure of increased permeability with inflammation.

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# THE IMMUNOLOGICAL SPECIFICITY OF CHEMICALLY ALTERED PROTEINS

## HALOGENATED AND NITRATED PROTEINS

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The immunological specificity of protein antigens has interesting aspects from the chemical viewpoint. Protein specificity appears to be closely associated with, and dependent on, chemical differences in the protein molecules. In most instances these differences are such that it is not possible to distinguish between similar proteins of different species by the usual methods of chemical investigation, but there are instances in which such a differentiation is possible by chemical or physical as well as by immunological tests. The differences which are responsible for serological specificity may not necessarily involve variations in the amount of certain special amino acids or even of free groups such as amino or carboxyl groups, but may be due merely to differences in the structural or spatial arrangement of certain groupings. A fundamental difference between antigenicity and specificity is indicated by the more recent work on complex antigens and haptens.

There are several methods applicable to the study of the relationship between immunological specificity and the chemical properties of antigens; (a) alterations in the chemical constitution of the protein can be effected by various chemical processes such as oxidation, iodination, nitration, esterification, etc. (Obermayer and Pick (1), Landsteiner (2)), (b) a comparison can be made of the immunological properties of closely related vegetable proteins (Wells and Osborne (3)) or animal proteins (cf. review by Wells (4) pp. 68-74), and (c) compounds of well-defined chemical nature can be attached to the protein molecule (Landsteiner (5)). Each of these methods has a special

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value in furnishing evidence about the nature of the groups which are concerned with specificity. The third method permits a study to be made of the effect produced by the introduction into the protein molecule of substances of related constitution and configuration, and many variations can be made in the constitution of the substituent group. The first method, the study of the specificity of chemically altered proteins, does not offer the same widespread opportunities since the methods of producing chemical changes in the protein molecule are rather limited; in addition there are few, if any, instances which cannot be objected to on the ground that the treatment to which the protein is subjected may produce other alterations besides that desired, for it is hardly possible to state with absolute certainty that the protein molecule can be attacked in such a way that only one significant change in the molecule occurs.

The first observations on the specificity of chemically altered proteins were made by Obermayer and Pick (1). The most significant changes were observed when serum was subjected to the action of iodine, nitric acid and nitrous acid and the so-called iodo-, nitro (xantho-) and diazo-proteins were obtained. These protein compounds still possessed antigenic properties but the chemical treatment had resulted in a loss of species specificity and the acquisition of a new specificity. Thus an iodoprotein from horse serum produced in a rabbit antibodies which reacted with iodized horse serum and with an iodoprotein derived from the serum of any species but not with native horse serum. Nitrated horse serum produced antibodies for any nitrated protein but not for horse serum or for iodized serum proteins. From these experiments the conclusion was reached by Obermayer and Pick (cf. also Pick (6)) that the species specificity of serum proteins is fundamentally related to the aromatic radicals present in the protein molecule and that any change in the aromatic nucleus destroys the original specificity and endows the molecule with a structural specificity characteristic for the new structure. Little evidence was available at that time however, that the action of iodine, nitrous acid or nitric acid was directed solely towards the aromatic amino acids, in particular the tyrosine molecules, and whilst these reagents are known to effect changes in the benzene ring, there may be other processes such as oxidation and hydrolysis which occur simultaneously. The methods of iodination used by Obermayer and Pick, iodination in hot acid solution (Hofmeister (7)) and iodination by the method of Blum and Vaubel (8) which involves treatment of the serum with iodine in the presence of sodium bicarbonate and heating at 50° for some time, will produce changes in the protein molecule other than simple iodination in the benzene ring. Also, in the preparation of nitrated and diazotized proteins, the treatment with acid may effect changes such as denaturation or hydrolysis.

Although in these three processes (iodination, nitration and diazotization) a distinct change occurs in the aromatic radicals of the protein, notably the tyrosine, the rest of the protein molecule is undoubtedly affected by the treatment.

One of the most interesting of these chemically altered proteins is the iodoprotein and several authors have obtained general confirmation of the observations made by Obermayer and Pick that treatment with iodine destroys, more or less completely, the original species specificity and occasions a new specificity.

Wells (9) obtained inconstant results with iodized serum in anaphylactic experiments with guinea pigs but with one preparation found that the iodized serum possessed the property of sensitizing guinea pigs to either the iodized serum or normal homologous serum. In general, however, no loss of species specificity occurred on iodination. Freund (10) carried out experiments similar to those of Obermayer and Pick and obtained confirmatory evidence that the specific complex of the protein is bound to the aromatic groupings of the molecule. Anaphylactic experiments have been carried out by various authors (11, 12, 13, and 14). Bauer and Murschhauser (15) described immunization experiments with iodized serum and iodized casein and conclude that only the labile proteins, such as serum proteins, lose their specificity on iodination and that the less labile proteins (cf. caseinogen) do not behave in this way.

The serological properties of nitrated proteins have recently been discussed by Ottensooser and Strauss (16).

There appears to be satisfactory evidence that the process of iodination leads to a more or less complete loss of species specificity and to the acquirement of a new specificity for iodinated proteins. It appeared desirable however, that more evidence should be available as to the nature of the changes involved in the iodination process before the loss of specificity is attributed solely to the introduction of iodine into the benzene ring of the tyrosine molecules present in the original protein. The view that the aromatic radicals largely determine the species specificity appeared to receive support from the fact that gelatin, which is deficient in aromatic amino acids, possesses no antigenic properties (Wells (9)). It is quite possible however, that the whole of the particular grouping or complex which is necessary for antigenicity may play little or no part in determining specificity, and indeed, the more recent observations on the haptens of pneumococci (Avery and Heidelberger) and the Forssman antigen (Landsteiner and Levene) indicate that the grouping which determines specificity may be entirely devoid of aromatic radicals. From the studies of the immunological properties of the iodoproteins and those of the nitrated proteins, it appeared probable, however, that the tyrosine groupings are concerned in some way with protein specificity, but the evidence upon which this view was based is not entirely convincing.

Several authors (Hofmeister (7), Hopkins (17), Blum and Vaubel (8) etc.) had observed that iodized proteins react negatively to Millon's test, a finding which appeared to justify the conclusion that substitution in the tyrosine nucleus occurs; but it was not until afterwards that Oswald isolated 3:5 diiodotyrosine from naturally occurring iodoproteins (18) and from artificially iodized proteins (19).

The methods of iodination used for the immunological experiments above mentioned—those of Hofmeister (7), and Blum and Vaubel (8)—may, as discussed above and as pointed out by Hofmeister, produce changes in the protein molecule in addition to simple substitution in the benzene ring of the tyrosine molecules. Iodization by the method of Blum and Vaubel leads, according to Blum and Strauss (20), to iodination in the tyrosine grouping, some oxidation processes and a change involving a destruction of one of the groups responsible for the biuret reaction. The latter authors also discuss the possibility of iodination in parts of the protein molecule which are unrelated to the tyrosine groupings. Very recently an investigation was made by Bauer and Strauss (20-a) on the chemical changes which occur in iodination and nitration of proteins. According to these authors nitration of proteins may involve a substitution in the tyrosine and tryptophane groups.

It seems desirable that a full investigation should be made of the serological properties of the halogenated and nitrated proteins with a view to gaining more information as to the rôle played by the aromatic groupings of the protein molecule in determining specificity. Consequently, halogenation or nitration has been effected by methods which have been modified in order to limit, as far as possible, the other changes such as hydrolysis and denaturation which readily occur in acid and alkaline solutions. For halogenation, bromine has been used in addition to iodine, and a study has been made of the serological properties of the brominated proteins.

## EXPERIMENTAL

### *Preparation of Antigens*

#### *Iodinated Serum Proteins.*

(a) This method was a modification of that of Blum and Strauss (21). Five hundred cubic centimeters of horse serum, 500 cc. of water and 500 cc. of 7%  $\text{NH}_4\text{OH}$  were mixed well and  $\text{N}/10$  iodine solution added, with shaking, in quantities of about 50 cc. at each time. The iodine solution was added until free iodine was detectable by starch-iodide paper 5 minutes after the last addition of iodine, and then acetic acid was added to give maximum precipitation. The solution was centrifuged and the precipitate was dissolved in about 1500 cc. of distilled water by the aid of a little dilute  $\text{Na}_2\text{CO}_3$  solution, the pH of the solution being kept below 8.5. The iodoprotein was reprecipitated twice more from a very faintly alkaline solution by acetic acid and then dissolved in 0.9%  $\text{NaCl}$  solution by the aid of a little dilute  $\text{Na}_2\text{CO}_3$  solution. The reaction of the solution was adjusted to pH 7.5 and sufficient phenol to give 0.25% was added to that part of the solution required for injection. The horse serum iodoprotein, reprecipitated several times

from slightly alkaline solution by acetic acid and washed well with alcohol, contained 9.0% of iodine. Similar preparations were made with beef, chicken and rabbit serum. All the iodoproteins prepared by this method reacted negatively to Millon's test.

(b) *Iodination in Hot Acid Solution.* ((7.1) cf. Landsteiner and Prášek (22).) The brown product was dissolved as far as possible in dilute sodium carbonate solution and reprecipitated with dilute acetic acid. The product was then redissolved in 0.9% NaCl by the addition of a small amount of dilute  $\text{Na}_2\text{CO}_3$ . The reaction of the solution was adjusted to pH 7.5. This preparation and the corresponding antisera were used for the experiments recorded in table IX only.

*Brominated Serum Proteins.* A modification of the method of Hopkins and Pinkus (23) was found most satisfactory for the purpose. A mixture of 250 cc. of horse serum and 500 cc. of distilled water was treated with bromine water until free bromine could be detected after the solution had been well shaken and allowed to stand for a few minutes. One per cent sodium carbonate solution was then carefully added to give maximum precipitation and the solution centrifuged. The precipitate was dissolved in about 1 liter of water with the aid of a small amount of dilute  $\text{Na}_2\text{CO}_3$ , the pH being kept below 8.5 and the bromoproteins precipitated by the addition of a small amount of 10% acetic acid. This precipitate was dissolved in about 300 cc. of 0.9% NaCl by the addition of a little dilute  $\text{Na}_2\text{CO}_3$ , the reaction of the solution adjusted to pH 7.5, and sufficient phenol to give 0.25% was added to the solution used for animal injections. The brominated horse serum proteins, purified by several precipitations with acetic acid, washed with alcohol and dried, contained 4.7% of bromine. The brominated sera reacted negatively to Millon's test. Attempts were made to modify this process by brominating in approximately neutral solution by adding  $\text{Na}_2\text{CO}_3$  or NaOH after the addition of bromine to the horse serum but the products obtained differed from that obtained by the method described above in their insolubility in neutral or faintly alkaline solution (pH 7.5 to 8.0).

*Chlorinated Serum Proteins.* A method analogous to the bromination method was used and a product obtained which had properties similar to those of the brominated proteins. A fairly considerable amount of chlorine was absorbed during the chlorination process but when the crude chloroproteins had been purified by several reprecipitations from slightly alkaline solutions with acetic acid and had been washed well with alcohol and then dried, they contained no combined chlorine. The results of the immunization experiments with the chlorinated proteins are, therefore, not included here since a comparison with the other halogenated proteins is not possible. The results showed that some slight loss of the original species specificity had occurred.

*Nitrated Serum Proteins.*

(a) With nitric acid ("xantho" proteins)—cf. Landsteiner and Prášek (22).

(b) With tetranitromethane ("nitro" proteins). One hundred cubic centimeters of serum, 100 cc. of pyridin, 200 cc. of distilled water and 30 cc. of tetranitromethane were mixed and shaken at intervals at room temperature. The mix-

ture assumed a reddish orange color which finally became orange brown, sometimes with the intermediate formation of a deep green colour. Further additions of pyridine were made to keep the solution neutral or faintly alkaline. The solution was centrifuged, after the addition in some cases of a small amount of dilute acetic acid to complete the precipitation, and the precipitate washed with about 200 cc. of acetone, centrifuged and washed twice more with about 200 cc. of acetone. The yellow precipitate was then suspended in water and dilute sodium carbonate added to render slightly alkaline. NaCl was added to give 1%, and the pH adjusted to 7.5. Sufficient phenol to give 0.25% in the solution was added to the suspension of nitrated horse serum used for the injections. The amount of protein in the filtered solutions was 0.5–1.5%.

*Metaprotein.* Twenty cubic centimeters of serum were mixed with 200 cc. of distilled water and 2 cc. of concentrated HCl (spec. grav. 1.18). The mixture was allowed to stand at 37°C. for 24 hours and the metaprotein precipitated by the addition of dilute sodium carbonate. The precipitate was washed with a little 0.9% NaCl solution and dissolved in water with the aid of a little dilute Na<sub>2</sub>CO<sub>3</sub> or very dilute NaOH. The solution had to be kept slightly alkaline to prevent precipitation of the metaprotein. Thymol was added as a preservative.

*Diazotized Serum Proteins.* (cf. Landsteiner and Prášek (22).)

### *Immunization*

Five rabbits received intravenous, or in some cases intraperitoneal, injections of the antigen at weekly intervals and the sera were tested 7 days after the third and, where necessary, the subsequent injections. The antigens used for injection were prepared from horse serum in every case.

*Iodo-horse Protein.* Five cubic centimeters of a solution containing 4.5% of iodoprotein were injected intravenously on the first occasion. Subsequent injections were made intraperitoneally since several fatalities occurred on the second intravenous injection. Two rabbits gave good antisera after three injections and two more after four injections.

*Acid Iodinated Protein.* (Hofmeister's preparation.) Two intravenous injections were made of 5 cc. of a solution containing 1.5% of iodoprotein, followed by intraperitoneal injections of 10–15 cc. of the solution at the subsequent injections. Four to six injections were necessary.

*Brominated Protein.* Intravenous injections were made of a solution containing 4% of bromoprotein. Two animals were bled after 3, one after 4 and one after 5 injections.

*Nitrated Protein* ("Xanthoprotein"). Intravenous injections were given of 5 cc. of a solution containing 2% of xanthoprotein. One animal bled after 3, one after 4, and one after 5 injections.

*Nitrated Protein* ("Nitroprotein") Intraperitoneal injections were made of 10 cc. of a suspension containing 2.5% of nitroprotein. Four animals bled 7 days after the third injection.

*Precipitin Tests*

For these tests the antigen solutions were diluted to 1:100, 1:500, 1:2500 and in certain cases 1:12500 in terms of a 5% stock solution, and 0.2 cc. used for each test. Two drops of the immune serum were added to each tube, the solutions shaken and readings made after 1 hour at room temperature (25 to 30°C.). The intensity of the reaction was indicated as follows:—(no reaction), f. tr. (faint trace), tr. (trace),  $\pm$ , +,  $+\pm$ ,  $++$ , etc.

The first readings in the following tables are those recorded after 1 hour at room temperature and the second readings after 2 hours at room temperature and overnight in the ice box. In most of the tables the results of tests with two antisera only, and in certain instances with only one serum, are recorded. Other antisera gave similar results and in each case at least three antisera were tested.

The iodoprotein preparation and the iodoprotein antisera used for the following experiments, except those recorded in table IX, were those corresponding to the first method of iodination, namely, iodination in the presence of ammonium hydroxide.

The serological properties of the iodinated and brominated proteins were investigated by experiments the results of which are given in tables I, II, and III. From table I it will be seen that the iodoprotein antiserum reacts to the maximum extent with iodinated serum and somewhat less with brominated sera. The bromoprotein antisera react with brominated or iodinated proteins, to a slightly greater extent with the former. Iodination and bromination lead therefore to a more or less complete loss of the original species specificity (tables II and III), but the reaction between an iodo-horse antiserum and the homologous antigen is always more marked than that with any other iodinated proteins. The same holds true for the brominated proteins, and in all cases there seems to be a retention of some species characteristics. The effect is more pronounced when the readings are made after a shorter interval of time, e. g., 5 minutes after mixing.

The results recorded in table IV and V show that the proteins nitrated by the two methods used have similar serological properties and that nitration by either method leads to the acquirement of a new characteristic specificity.

Cross tests made between nitrated and halogenated proteins (table VI) indicate that some interaction occurs between certain halogenated proteins (in the main the brominated products) and antisera to nitrated proteins. In these cases there is no question of a species reaction since

TABLE I

Antigen	Antigen dilution	Iodo-horse antiserum 1635				Iodo-horse antiserum 1643				Bromo-horse antiserum 1822				Bromo-horse antiserum 1823			
Iodo-horse	1:100 1:500 1:2500	++± +± ±	++± +± +	++± +± ±	++ +± +	++± +± +	++± +± +	++± +± +	++± +± +	++± +± +	++± +± +	++± +± +	++± +± +	++± +± +	++± +± +		
Iodo-beef	1:100 1:500 1:2500	++± +± tr	++± +± ±	++± +± ±	++± +± tr	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±		
Iodo-chicken	1:100 1:500 1:2500	++± +± tr	++± +± ±	++± +± ±	++± +± tr	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±		
Iodo-rabbit	1:100 1:500 1:2500	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±	++± +± ±		
Bromo-horse	1:100 1:500 1:2500	++± + tr	++± + ±	++± + ±	++± + -	++± + ±	++± + ±	++± + ±	++± + ±	++± + ±	++± + ±	++± + ±	++± + ±	++± + ±	++± + ±		
Bromo-beef	1:100 1:500 1:2500	++± ± tr	++± ± ±	++± ± ±	++± ± -	++± ± ±	++± ± ±	++± ± ±	++± ± ±	++± ± ±	++± ± ±	++± ± ±	++± ± ±	++± ± ±	++± ± ±		
Bromo-chicken	1:100 1:500 1:2500	++ + f. tr	++ + tr	++ + tr	++ + -	++ + ±	++ + -	++ + -	++ + -	++ + -	++ + -	++ + -	++ + -	++ + -	++ + -		
Bromo-rabbit	1:100 1:500 1:2500	++± + tr	++± + ±	++± + ±	++± + -	++± + ±	++± + -	++± + -	++± + -	++± + -	++± + -	++± + -	++± + -	++± + -	++± + -		

the antigens tested were prepared from proteins different from that which gave rise to the antiserum. A similar cross reaction occurs between bromo-horse antibodies and nitrated proteins antigens (cf. table VII).

Table VIII gives the results of experiments with diazotized proteins and shows that diazoproteins and nitrated proteins are very closely related serologically. Thus diazo-beef and nitro-beef proteins, for

TABLE II

Antigen	Antigen dilution	Iodo-antiserum						Bromo-antiserum		
		1657		1635		1643		1822	1823	1821
Horse serum	1:100	±	+	—	f.tr.	—	—	—	—	—
	1:500	±	+	—	f.tr.	tr.	tr.	—	—	—
	1:2500	tr.	±	—	f.tr.	f.tr.	tr.	—	—	—
	1:12500	—	—	—	—	—	—	—	—	—

TABLE III

Immune serum	Antigen dilution	Antigen					
		Iodo-horse		Bromo-horse		Horse serum	
Anti-horse No. 55	1:100	f.tr.	f.tr.	—	—	+++	+++±
	1:500	tr.	tr.	—	—	++	++
	1:2500	—	—	—	—	±	+
Anti-horse No. 1807	1:100	—	±	—	—	++	++±
	1:500	—	tr.	—	—	+	+±
	1:2500	—	—	—	—	±	+

example, react equally well with antisera to any type of nitrated protein investigated. Diazotized proteins, like nitrated proteins, also give some cross reactions with brominated proteins.

In table IX are given the results of experiments with the proteins iodinated in acid solution (Hofmeister's preparation). These antigens do not appear to be very active serologically but give antisera which react quite well with iodinated proteins prepared by the more gentle treatment (method (a)) or with brominated proteins. The antigens obtained by iodination in acid solution gave, however, remarkably



weak reactions with antisera to iodoproteins prepared by the action of iodine in ammoniacal solution.

Since it has been shown (Landsteiner (24), Landsteiner and van der Scheer (25)) that the precipitin reaction of azo-proteins can be inhibited specifically by simple substances containing the specific reactive grouping, it was thought possible that similar tests would give some

TABLE IV

Immune serum	Antigen dilution	Antigen							
		Xantho-horse		Xantho-beef		Xantho-chicken		Xantho-rabbit	
Xantho-horse No. 1770	1:100	+	+±	±	±	tr.	±	tr.	±
	1:500	+±	++	+	+±	+	+±	+	+±
	1:2500	+	+	±	+	tr.	+	±	+
Xantho-horse No. 1766	1:100	+±	++	±	+	+	+	±	+
	1:500	+	++	+	+±	+	+±	+	+±
	1:2500	±	+	±	+	±	+	±	+
Nitro-horse No. 1879	1:100	++	++±	±	+	tr.	±	tr.	tr.
	1:500	++	++±	+	+±	+	+±	+	+±
	1:2500	+	+±	±	+	±	+	+	+
Nitro-horse No. 1881	1:100	++	++±	+	+±	+	+±	+	+±
	1:500	+±	++	+	+±	+	+	+	+
	1:2500	±	+	tr.	+	tr.	+	±	+
Anti-horse No. 1807	1:100	—	—	—	—	—	—	—	—
	1:500	—	—	—	—	—	—	—	—
	1:2500	—	—	—	—	—	—	—	—

The term "xanthoprotein" is used here to refer to the nitrated proteins obtained by treatment with nitric acid and "nitroprotein" for the products obtained by the tetranitromethane method.

indication as to the nature of the reacting groups of the halogenated proteins.

Inhibition tests with equimolecular quantities of various substances (table X) show that whereas tyrosine and several other amino acids have no inhibitory effect on the reaction between halogenated proteins and the corresponding antisera, 3:5 diiodo-, 3:5 dibromo- and 3:5 dichloro-tyrosine have a very marked inhibitory influence on the

TABLE V

Immune serum	Antigen dilution	Antigen					Horse serum
		Nitro-horse	Nitro-beef	Nitro-chicken	Nitro-rabbit		
Xantho-horse No. 1770	1:100	+	+	+	+	+	+
	1:500	+	+	+	+	+	+
	1:2500	f. tr	+	f. tr.	f. tr	+	+
Xantho-horse No. 1764	1:100	+	+	+	+	+	+
	1:500	+	+	+	+	+	+
	1:2500	tr	+	tr	+	+	+
Nitro-horse No. 1879	1:100	+	+	+	+	+	+
	1:500	+	+	+	+	+	+
	1:2500	+	+	+	+	+	+
Nitro-horse No. 1881	1:100	+	+	+	+	+	+
	1:500	+	+	+	+	+	+
	1:2500	+	+	+	+	+	+
Anti-horse serum No. 1807	1:100	-	-	-	-	-	+
	1:500	-	-	-	-	-	+
	1:2500	-	-	-	-	-	+

See note at the bottom of table IV for an explanation of the terms xanthoprotein and nitroprotein.

iodoprotein or bromoprotein precipitin reaction. It is interesting to note that the diiodo-compound is most active in this respect and the dichloro-compound least active. Stronger solutions of all three dihalo-

TABLE VI

Immune serum	Antigen dilution	Antigens					
		Iodinated serum				Brominated serum	
		Beef		Chicken		Beef	Chicken
Xantho-horse No. 1770	1:100	—	—	—	—	±	+
	1:500	—	—	—	—	±	±
	1:2500	—	—	—	—	—	tr
Xantho-horse No. 1766	1:100	—	±	—	tr	±	+
	1:500	—	tr	—	tr	±	±
	1:2500	—	f.tr	—	f.tr	tr	tr
Nitro-horse No. 1879	1:100	tr	±	—	tr	±	+
	1:500	tr	+	—	tr	tr	±
	1:2500	f.tr	tr	—	—	f.tr	tr
Nitro-horse No. 1883	1:100	—	tr	—	—	±	+
	1:500	—	tr	—	—	±	±
	1:2500	—	—	—	—	—	tr

TABLE VII

Antigen	Antigen dilution	Antigens					
		Xantho-proteins				Nitrated proteins	
		Beef		Chicken		Beef	Chicken
Iodo-horse No. 1635	1:100	—	—	—	—	—	—
	1:500	—	—	—	—	—	—
	1:2500	—	—	—	—	—	—
Bromo-horse No. 1823	1:100	—	—	—	—	tr	±
	1:500	—	—	—	—	f.tr	±
	1:2500	—	—	—	—	—	f.tr
Anti-horse serum No. 1807	1:100	—	—	—	—	—	—
	1:500	—	—	—	—	—	—
	1:2500	—	—	—	—	—	—

generated tyrosine compounds inhibit completely or almost completely the reaction between iodoprotein antigen and iodoprotein antiserum, and that between the brominated protein antigen and bromo-protein



TABLE IX

Immune serum	Antigen dilution	Antigen										
		Iodoproteins (Hofmeister's preparation)					Iodoproteins					
		Horse	Beef	Chicken	Rabbit	Iodoproteins		Bromoprotein	Horse serum			
						Horse	Beef					
Anti-iodoprotein (Hofmeister's preparation) No. 1771	1:100	±	tr	±	—	f. tr	++	++	±	+	+	—
	1:500	±	f. tr	±	—	±	++	++	±	±	+	—
	1:2500	—	—	f. tr	—	tr	+	++	+	±	+	—
Anti-iodoprotein (Hofmeister's preparation) No. 1772	1:100	±	f. tr	tr	—	—	++	++	±	+	+	—
	1:500	tr	—	+	—	tr	++	++	±	+	±	—
	1:2500	—	—	f. tr	—	f. tr	+	++	+	tr	+	—
Anti-iodo-horse No. 1643	1:100	—	—	tr	—	—	++	++	+	+	+	—
	1:500	—	—	—	—	—	++	++	±	+	+	—
	1:2500	—	—	—	—	—	+	++	±	+	+	—

TABLE X

*Inhibition Tests.* 0.2 cc. of a solution of the substance tested for inhibiting action (containing 0.5 millimol per 100 cc. of 0.9% NaCl in each case) + 0.05 cc. of the antigen solution + 2 drops of immune serum. The strength of the antigen solution for the first four horizontal columns was 1:100 and for the others 1:20, of a 5% stock solution.

Antigen	Immune serum	Control	Substances tested for inhibitory action									
			Tyrosine	3.5 diiodo-tyrosine	3.5 dibromo-tyrosine	3.5 dichloro-tyrosine	Alanin	Phenylalanin	Histidin	Phenol	o-Iodophenol	Potassium iodide
Horse serum	Horse serum No. 1807	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±
Xantho-chicken	Xantho-horse No. 1770	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±
Iodo-chicken	Iodo-horse No. 1635	+ + ±	+ + ±	-	tr	±	+ ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±
Bromo-chicken	Bromo-horse No. 1822	+ + ±	+ + ±	-	f. tr	tr	±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±
Bromo-chicken	Iodo-horse No. 1635	+ + ±	+ + ±	-	tr	tr	±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±
Iodo-chicken	Bromo-horse No. 1822	+ + ±	+ + ±	-	-	f. tr	f. tr	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±

antiserum or the cross reactions between iodoproteins and bromoproteins. None of these inhibiting substances have any influence on the reaction between nitrated proteins and the corresponding antisera or on the reaction between horse serum antiserum and the homologous antigen, indicating that the inhibiting effect of 3:5 dihalogenated tyrosine on the iodoprotein and bromoprotein precipitin reactions is specific.

All the antisera were tested with metaprotein antigens (beef and chicken) but in no case was any positive reaction noted.

#### DISCUSSION

The results of the experiments with halogenated and nitrated proteins undoubtedly offer support for the view that the aromatic radicals of the protein molecule may be concerned with the determination of specificity; but there are changes in the protein molecule which do not appear to be concerned with the aromatic nuclei but which lead to a loss of species specificity. Such an effect is produced by esterification with acid alcohol, acetylation, or methylation (Landsteiner and Prášek (26), cf. also (27, 28)).

Whilst the methods previously used were rather drastic in nature, the methods of halogenation employed here were such that hydrolytic and oxidative changes were reduced as far as possible.

Iodination and bromination result in a more or less complete loss of the original species specificity and a new specificity characteristic of halogenated proteins is produced. The injection of iodinated horse serum into a rabbit gives rise to the formation of antibodies which react with iodinated heterologous sera or with the brominated sera. Similar antibodies are produced by the injection of brominated serum and in these antigens there appears to be some common active grouping which is responsible for the new specificity. This new grouping seems to be bound up with the tyrosine molecules and for several reasons it appears probable that the serological properties of the halogenated protein antigens are due to the disubstitution of halogen in the 3:5 position of the tyrosine molecules. Ample evidence exists for the view that iodoproteins contain the 3:5 diiodotyrosine structure (Oswald (18, 19), Blum and Vaubel (8)), but in addition the possibility that substitution occurs in other places, (e. g. in the histidine groups), must not

be overlooked. According to Blum and Strauss (21), however, gentle iodination in the presence of ammonium hydroxide does not result in any appreciable attachment of iodine to the histidine nitrogen atoms.

Iodination or bromination leads to a loss of the power to give a positive Millon's reaction and this loss is attributed to disubstitution of halogen in the tyrosine ring in the ortho positions relative to the hydroxyl group (Blum and Vaubel (8)). This disappearance of a positive Millon's reaction runs parallel in the experiments described here, with the loss of the original species specificity, and incomplete iodination results in the formation of a product which still gives Millon's reaction and retains some of the species specificity. More convincing proof of the view that the new specificity of halogenated proteins is dependent upon the presence in the protein of 3:5 dihalogenated tyrosine groupings is derived however from the inhibition tests, since 3:5 diiodotyrosine has a marked inhibitory effect on the reaction between iodoproteins and the iodoprotein antiserum whereas tyrosine and other amino acids have none. Thus the iodoprotein antibodies have some active grouping which has a special affinity for the 3:5 diiodotyrosine grouping and indeed for any 3:5 dihalogenated tyrosine structure, since the corresponding bromo- and chloro-derivatives of tyrosine have a similar combining capacity for iodoprotein, or bromoprotein antibodies. These inhibitory reactions appear to be specific, and from other evidence (Landsteiner (24)) it seems possible that reactions of this type will be useful for the determination of the structure or configuration of chemically altered antigens.

The results of the experiments with nitrated (or xantho) proteins confirm the findings of Obermayer and Pick (1), that after nitration with concentrated nitric acid there is a more or less complete loss of species specificity with the production of a new specificity characteristic for nitrated proteins. This method of nitration undoubtedly results in fairly drastic hydrolytic, and possibly oxidative, changes, and hence it was considered desirable that some other method of nitration of proteins, preferably one in which strong acids are not used, should be devised. The new method adopted, a method based on that of Schmidt and Fischer (29) and involving nitration with tetranitromethane in neutral or slightly alkaline solution, yields nitroproteins which resemble, in general chemical properties and immunological



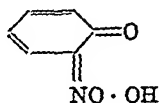
reactions, those obtained by the nitric acid method. Thus the anti-serum to nitrated horse serum (tetranitromethane method) reacts with any nitrated serum or with the xanthoproteins (nitric acid method) from any serum, and the nitrated proteins react with the xanthoprotein antiserum.

A reinvestigation of some of the immunological properties of diazotised proteins has confirmed the observations of Landsteiner and Prášek (22) rather than those of Obermayer and Pick (1). The latter authors imply that xanthoprotein and diazoprotein are serologically well differentiated on account of the different nature of the substituents, and they conclude that substitution of a diazo group, presumably in the benzene ring, leads to a new structural specificity. Landsteiner and Prášek, however, found that the xanthoproteins and diazotised proteins show close serological relationship; thus the diazo antigens and the xantho antigens reacted almost equally well with diazotised protein antiserum or with xanthoprotein antiserum. In the experiments described here, diazotised serum proteins react like the xanthoproteins or the nitrated proteins and can hardly be differentiated in precipitation tests with xanthoprotein and nitrated protein immune sera. These facts seem to indicate that the diazotized protein and the nitrated protein have constitutions which are closely related.

Diazotization of proteins by the action of nitrous acid has proved difficult to account for chemically, since, according to all the evidence available, proteins contain no aromatic amino groups. Richard (30), who first described the reaction, reached the conclusion that proteins must contain aromatic amino groups, but another interpretation was advanced by Landsteiner (31) who showed that treatment of aromatic oxyacids, such as salicylic acid or tyrosine, with nitrous acid leads to the formation of diazo compounds (cf. also Weselsky (32), Morel and Sisley (33)). One may conclude that a similar process occurs when proteins are treated with nitrous acid, and that a diazo group is introduced into the tyrosine nucleus, probably in the ortho position to the hydroxyl group.

It is rather remarkable that diazotized salicylic acid and the diazotized proteins have a distinct yellow colour, comparable with that of the nitrated proteins. The marked yellow colour of the ortho-

nitrophenols has been attributed by Armstrong (34) to a quinoid structure (cf. also Hantzsch (35)).



This explanation might reasonably be extended to the case of the nitrated proteins and it is not improbable that a similar structure exists in the diazotized phenolic compounds or diazotized proteins. Such a change in the aromatic nucleus of the tyrosine molecule which leads to the formation of a quinoid structure might account for the serological alteration effected by nitration or diazotization of proteins. This view would offer a reasonable explanation for the close serological similarity between nitrated and diazotized proteins. At any rate it appears that a considerable difference in the nature of the substituent grouping has, in this case, no influence on the serological specificity.

The results of the work of Obermayer and Pick are generally accepted as a proof that the serological differences observed by the authors are merely due to chemical differences in the radicals introduced into the protein molecule. In the present investigation, however, an intense cross reaction between nitrated proteins and diazotized proteins and a strong interaction between iodo- and bromoproteins, were observed. Apart from slight cross reactions there was a sharp serological distinction between the nitrated and diazotized protein on the one hand and the halogenated protein on the other but this difference is not necessarily dependent upon the nature of the substituents only. It may depend on the place of substitution and on the number of entering groups which is probably different in halogenated and nitrated proteins (cf. 19, 36, 20-a).

It is not possible from the evidence available at the present time,—and to acquire more will be difficult,—to decide how far the introduction of different radicals into the same position of the aromatic nucleus would lead to serological differentiation. Thus it would be of interest to know whether the mere replacement of halogen atoms of halogenated proteins by, for example, hydroxyl, nitro, or methyl groups or by more complex radicals, would lead to the formation of antigens which would no longer react with antibodies to the halogenated pro-

tein. From other evidence, especially in regard to the azo-proteins (Landsteiner and van der Scheer (37)) it has been found that various substituents situated in the same position in the benzene ring do not necessarily occasion pronounced differences in serological behaviour.

In all the experiments with halogenated and nitrated proteins it has been observed that the antisera to a chemically altered horse serum react to the maximum extent with the homologous antigen. These differences, although distinct, are not so pronounced as some of those previously noted in the case of the xanthoproteins by Landsteiner and Prášek (22). These observations tend to indicate that the species characteristics are not entirely destroyed by nitration or halogenation, although the power to react with an immune serum to the native homologous serum may be entirely or practically entirely lost.

One possible explanation of these facts would involve the assumption that more than one group in the protein molecule is responsible for the species characteristics and that chemical treatment such as halogenation or nitration does not change or destroy the whole of these groupings. It is also possible that several active groupings of the antigen molecule combine with a similar number of groupings in the corresponding antibody and thus an antigen would give rise to the formation of an antibody which has its combining groups orientated in a manner similar to the orientation of the specificity groupings of the antigen. Thus the maximum reaction will be obtained only when the spatial distribution of the combining group or groups of the antigen and antibody are closely related, i.e., when the antiserum reacts with the homologous antigen.

#### SUMMARY

The serological properties of iodoproteins prepared by a method which involves less drastic treatment of the protein than the methods previously used for this purpose confirm the findings of Obermayer and Pick (1) and later authors, that iodination of proteins results in a more or less complete loss of species specificity and that a new specificity characteristic for iodoproteins is produced.

A serological investigation of brominated proteins has been made for the first time. These preparations are only slightly different from iodized proteins in their serological properties.

Evidence is submitted which indicates that the radical in iodoproteins which is responsible for the specificity is not iodine but the 3:5 dihalogenated tyrosine grouping. Thus marked inhibition of the iodoprotein (or bromoprotein) precipitin reactions is effected by 3:5 dihalogenated tyrosine, not by iodophenol or potassium iodide.

A reinvestigation has been made of the serological properties of nitrated and diazotized proteins. Proteins nitrated by nitric acid, or by a method which does not appear to have been used for proteins hitherto, namely nitration with tetranitromethane in neutral or slightly alkaline solution, acquire a new common serological specificity. The nitrated proteins and diazotized proteins show, in confirmation of the results of Landsteiner and Prášek (22) and in contrast to the findings of Obermayer and Pick, very little difference in their reactions. Thus diazotized proteins and proteins nitrated by either of the two methods above mentioned react equally well with any nitroprotein antiserum. This interaction exists in spite of the difference in the substituents, either because the substitution with the nitro- or diazo-group occurs in the same position in the aromatic nucleus, possibly in the ortho position to the hydroxyl group, or because of some other structural similarity. In the last connection it is suggested that both compounds may have a quinoid structure as has been assumed for ortho-nitrophenols.

Whilst this assumption could account for the marked serological difference of nitrated and halogenated proteins it should also be mentioned that iodination (and bromination) lead to a disubstitution of halogen in the two ortho positions relative to the hydroxyl group of the tyrosine whereas nitration of proteins probably results in the formation of mononitrotyrosine and substitution in the tryptophane group as well (19, 36, 20-a). It is probably impossible therefore, to draw a strict analogy between nitration (or diazotization) and halogenation of proteins since a comparison of their immunological properties is not exactly a comparison of the effect of substituting a different group in the same position. Accordingly it would appear that as yet no definite conclusions can be drawn as to the serological effect of differences in the chemical nature of various substituents in the aromatic nucleus although some influence is likely for general reasons.

All of the chemically altered proteins still retain a small amount of

the original species specificity, and the antisera always react to a slightly greater extent with the homologous antigen than with similarly treated antigens prepared from heterologous sera. This difference occurs even when the possibility of some unaltered protein being present in the antigen can be practically excluded.

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## A CONCENTRATING DIALYZER

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(Received for publication, November 8, 1929.)

A device has already been described<sup>1</sup> for rapid and efficient dialysis of fluids placed in collodion bags. This device has been improved in several ways and may now be used not only to remove salts, sugars and other small molecules but also to concentrate simultaneously the remaining fluid to a small volume while keeping it at a low temperature and sterile. The improved dialyzer will be described below. It will concentrate a liter of an aqueous solution (such as whey or urine) to a few cubic centimeters (or complete dryness if desired) in 3 to 4 hours and, at the same time, remove the dialyzable materials, such as salts, sugars, etc. The apparatus is not intended to supplant devices for electrodialysis such as those designed by Bronfenbrenner and others, but to be used where electrodialysis is not desirable or not necessary.

### *Description of the Dialyzer*

In the new dialyzer the fluid is contained in collodion bags at atmospheric pressure while cold water is passed around the bags under negative pressure, and the apparatus is kept rocking. The rocking accelerates the dialysis by agitating the fluid. It also distributes the fluid over the whole surface of the membrane. In this way the outward passage of water is independent of the volume of fluid almost to the end.

In Fig. 1 will be seen a perspective view of the dialyzer connected up for use. It consists of a 32 by 7 inch rocking board (with 2 by 7 inch pieces on the ends) which is supported above a 17 by 34 inch baseboard by 9½ by 2½ inch supports. Two heavy screws, 7 inches above the baseboard, serve as a pivot for the rocking board while a windshield scraper keeps it in motion.

Fifteen pyrex glass cells are fastened on the rocking board. In these cells are

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<sup>1</sup> Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1928, 11, 641.



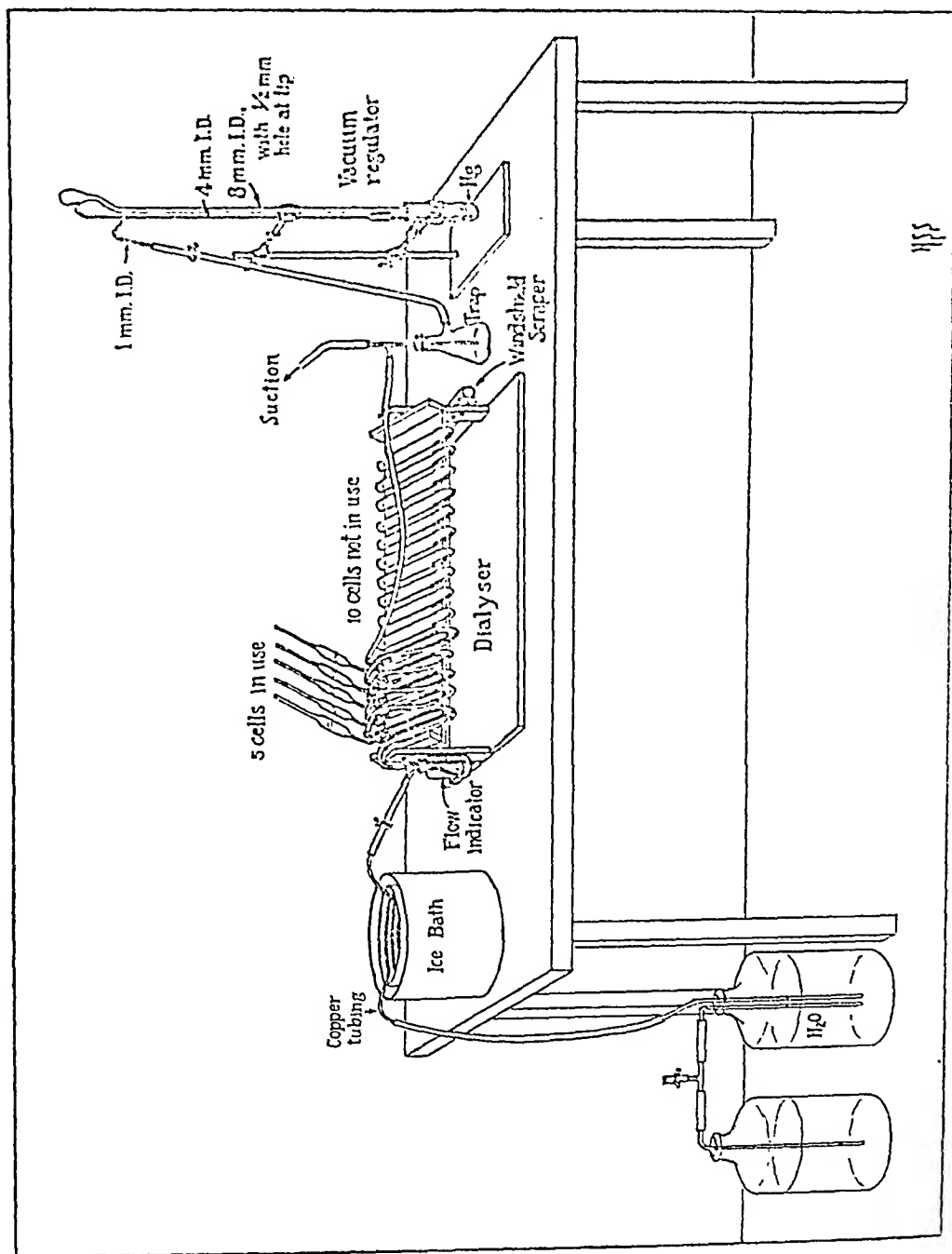


Fig. 1. Dialyzer in use

placed the collodion bags filled with the fluid to be dialyzed, each bag being connected with a pyrex reservoir bulb, as shown. Each bag holds 40 to 45 cc. of fluid, while 30 cc. more may be placed in each bulb. The details of the cells are shown more clearly in Fig. 2.

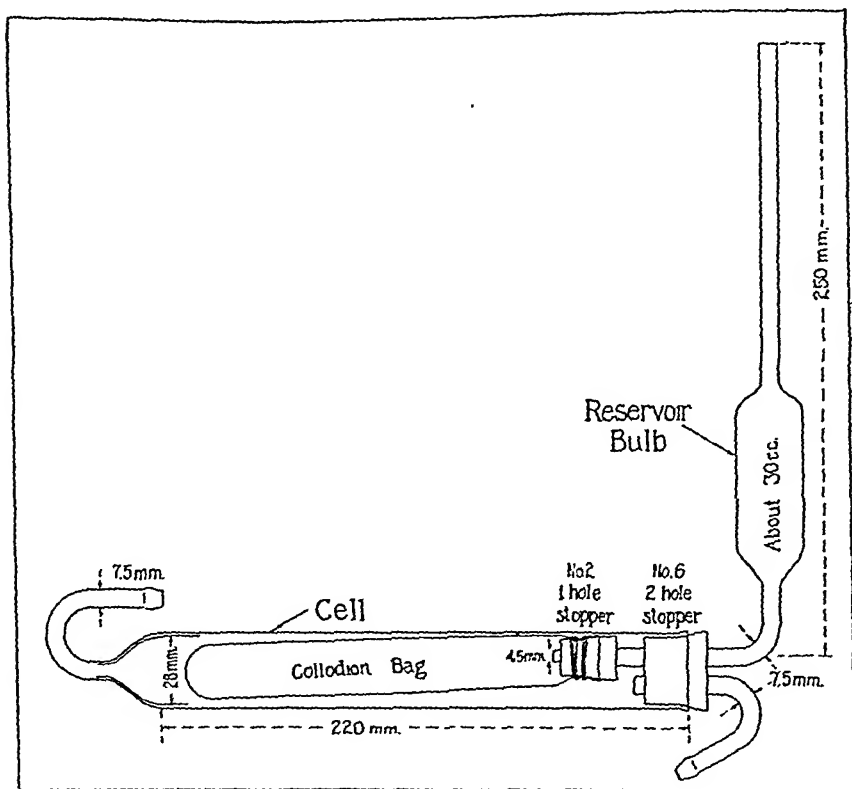


FIG. 2. Details of a cell and reservoir

The water (or salt solution) used for the "outside fluid" in dialyzing is placed in bottles on the floor and is sucked up, through an ice bath, into the apparatus where it passes first through the flow indicator and then successively through the 15 cells. The water then passes to a vacuum regulator and to a water suction pump. The vacuum regulator allows air to enter the system when the suction is too high. The ice bath keeps the dialyzed fluid below 10°C. For better cooling towels may be placed over the cells.

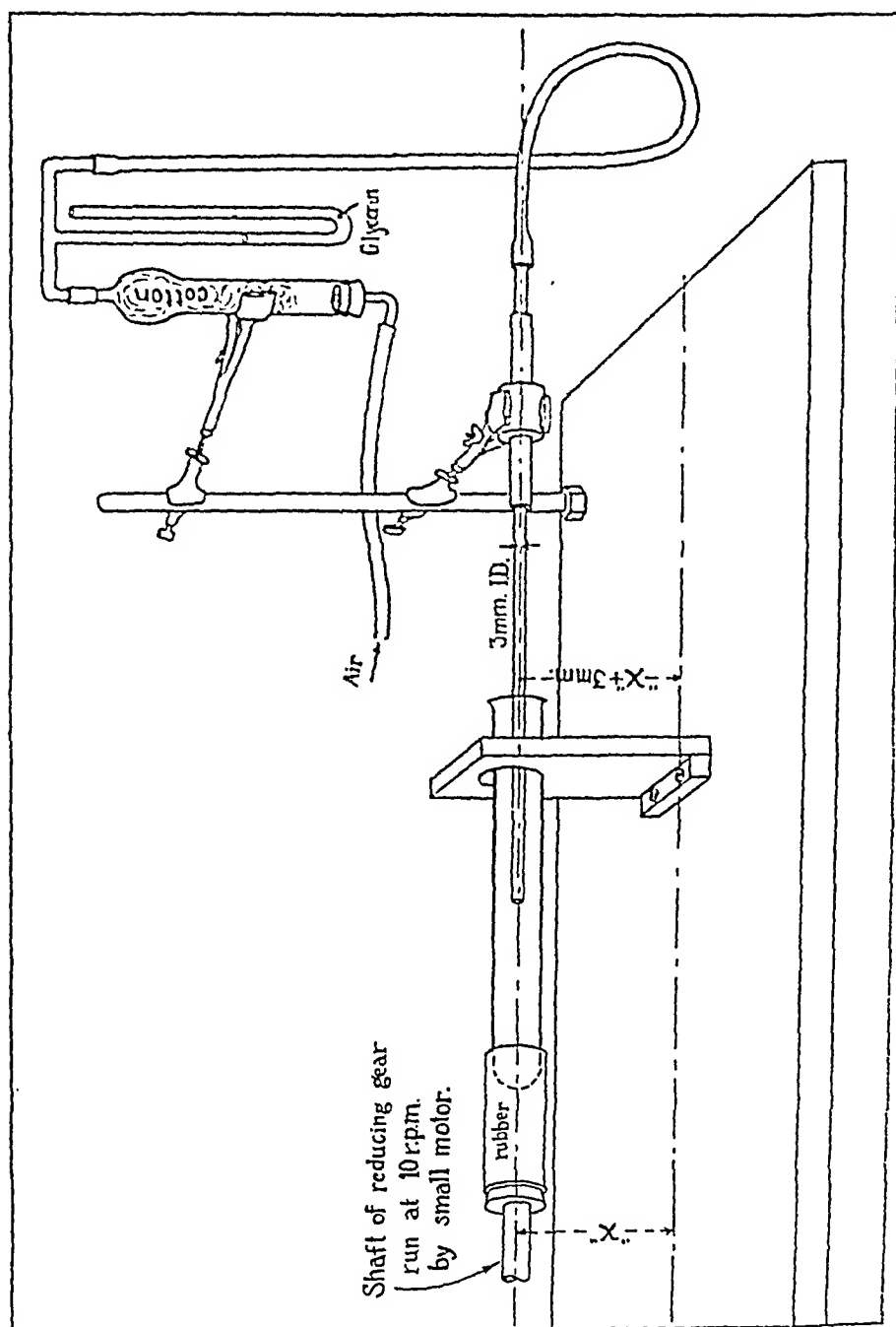


Fig. 3. Portion of machine for making collodion membranes?

*Making the Collodion Bags*

The method of making and testing the collodion bags is so important to the proper functioning of the apparatus that the technique is described in detail.

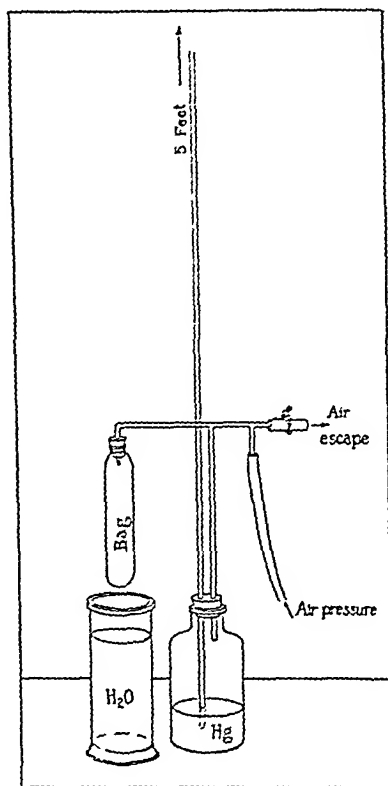


FIG. 4. *Bag Tester.* The air escape is gradually closed to increase the pressure in the bag. The cylinder of water is raised to immerse the bag.

The collodion bags should stand a test pressure of 70 cm. of mercury without leaking or breaking, and be so uniformly permeable that when filled with water and subjected to a 30 cm. pressure, the outside will immediately be completely covered with beads of water which has been forced through.

The membranes are made in pyrex test tubes 175 mm. long and 19 mm. inside diameter (each tube should be measured). 10 cc. of Merck's collodion is placed in a tube and the tube is turned three times by hand to wet the whole surface. It is

then placed in a machine (see Fig. 3) which rotates it in nearly a horizontal position 15 times a minute for 10 minutes.<sup>2</sup> The angle of the test tubes in the machine is such that the mouth is  $\frac{1}{8}$  inch higher than the closed end.

The rotation is then continued 10 minutes longer while air is blown through a glass tube inserted about half the length of the test tube. The pressure and volume of air are so regulated that there is a pressure of about 1 cm. of *glycerine* at a point beyond which the air must pass through 36 inches of rubber and glass tubing with a 3 mm. inside diameter. The blowing tube should *not* be constricted at the end, to form a nozzle; on the contrary it is better to flare it slightly, but this is not necessary.

The tubes containing the membranes, thus made, are then removed, filled with water and allowed to stand 15 minutes before the bags are taken out. Each bag is then trimmed, placed tight on a No. 2 one-hole rubber stopper, and fastened with a No. 8 rubber band, which is wound around it about four times.

The bags are next tested with the device shown in Fig. 4. It is connected up with an air pressure system and the screw-lamp on the escape tube is closed until the desired pressure is obtained in the bag, as indicated by the mercury manometer. The pressure should be increased very slowly. When it is seen that the bag does not break (at say 60 cm. of mercury) a cylinder of water is brought up around the bag until the latter is completely immersed. Small holes will be shown by a stream of bubbles.

The bags are then filled with 60 per cent alcohol and stoppered with pieces of glass rod, tapered at one end and flattened like a nail head at the other. If the bags are to be used the same day they are immersed in distilled water until required. If they are not to be used until the next day they may be immersed in 60 per cent alcohol overnight and placed in water the next morning. For best results they should be used within 24 hours but they can be kept for two or three days if necessary.

### *Use of the Dialyzer*

The bags are emptied by removing the plugs and repeatedly inverting them, to allow air to enter. Squeezing should be avoided. A second rubber band is then placed on each bag and the fluid to be dialyzed may be introduced with a pipette stuck into the hole of the stopper. Air is removed a little at a time by sucking on the pipette and then allowing the fluid to flow in. When a large number of bags

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<sup>2</sup> The machine in use can make two membranes at a time, one on each end of the shaft of a reducing gear. This is adapted from a machine made by J. H. Northrop and M. Kunitz (*J. Gen. Physiol.*, 1925-26, 9, 315) in which one membrane was made at a time. Dr. Anson has further improved the apparatus so that it makes five membranes at once. His five tubes are parallel to each other and are connected by gears.

are to be filled it is easier to siphon the fluid into the bags using a glass tip of about 2 mm. outside diameter pushed far enough through the stopper to allow the escape of air.

The Pyrex reservoir bulbs are sterilized in an autoclave, a plug of cotton having been placed in the upper end. They are inserted in two-hole, No. 6 rubber stoppers and then filled like a pipette after inserting the tip in a short piece of rubber tubing attached to a glass tube projecting into a flask of the fluid.

After both a bag and a bulb are filled (preferably by different people) the two are joined and inserted in a cell of the dialyzer which contains a little water. When all the cells are thus filled the rubber connections are made and the suction is turned on to slightly more than the desired vacuum. Adjustment is made by means of the regulator to a negative pressure of about 30 to 40 cm. of mercury. A pinch-cock between the water bath and flow indicator regulates the flow of water to about 8 cc. per minute per bag. In the flow indicator the water squirts upward at an angle and gives an approximate idea of the rate of flow.

When the cells are full of water, and everything is adjusted, the windshield scraper is turned on at about 10 cycles a minute, rocking from about 45° forward to 45° backward. This is continued until the fluid inside the bags is sufficiently concentrated.

The apparatus with 15 cells will hold about 650 cc. of fluid if only the bags are filled and should concentrate this to a thick syrup in 1 to 2 hours. If the reservoirs are also filled over a liter of fluid may be handled at once and require 3 to 4 hours. Additional fluid may be placed in the reservoirs during operation. No aqueous solution should require over 6 hours unless it contains material which clogs the colloid membranes.

Concentration may easily be continued to complete dryness but it is best to stop before the removal of fluid from the bags becomes too difficult. When it is desired to remove one or more bags (or if a bag should break) pinch-clamps are placed on the water tubing on both ends of the dialyzer and the desired changes are made. The concentrated fluid may be removed by inserting the tip of a pipette in the stopper of each bag and sucking until the bag is collapsed. With the technique used the fluid may be kept sterile throughout the dialysis.

#### SUMMARY

An apparatus is described for dialyzing aqueous solutions and simultaneously concentrating them to a thick syrup, or even to dryness.

The fluid is placed in collodion bags with reservoir bulbs open to atmospheric pressure. The bags are surrounded by flowing cold water, under negative pressure, and are subjected of a rocking motion. Over a liter of fluid can be concentrated to a few cubic centimeters in 3 to 4 hours. It is kept cold and sterile during the process.

# THE BACTERIAL GROWTH INHIBITOR (LACTENIN) OF MILK

## I. THE PREPARATION IN CONCENTRATED FORM

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(Received for publication, November 8, 1929)

A substance capable of inhibiting the growth of certain bacteria is known to exist in cow's milk. This substance was shown, in a previous paper,<sup>1</sup> to prevent multiplication of scarlet fever streptococci until all eventually die. The mastitis streptococcus, however, is restrained for only a few hours, then growth suddenly begins and continues with extreme rapidity. This phenomenon was attributed to the development of a type resistant to the inhibitor since in the first place the inhibitory substance was not destroyed by this action, and in the second place the resistant streptococci are not restrained when transferred to fresh samples of milk.

In this paper the above described substance will be called *lactenin*;<sup>2</sup> and the potency of a solution or material in this substance will be referred to as its *lactenic* activity.

The lactenic activity of whole milk is known not to be decreased by the removal of the fat or by the coagulation of casein with rennet. Whey contains about 6 per cent solids, most of which are sugar and salts, while 0.4–0.5 per cent is protein. With this material as a starting point we have attempted to obtain as concentrated and pure a preparation as possible.

## EXPERIMENTAL

In the following experiments milk was drawn directly from cleansed udders into sterile bottles and at once chilled. It was centrifuged and thus largely freed

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<sup>1</sup> Jones, F. S., and Simms, H. S., *J. Exp. Med.*, 1929, 50, 279.

<sup>2</sup> From the Latin: *lac*, milk; and *teneo*, hold.



from fat. The skimmed milk was always heated at 58°C. for 20 minutes and sufficient rennet solution added to insure coagulation. The rennet-milk mixtures were kept at 38°C. until about half the original volume could be withdrawn as whey. Later, when larger quantities were needed, the milk was pasteurized in centrifuge bottles, and after rennet coagulation, centrifuged at high speed.

Two methods were employed for test purposes. When the substances to be tested were regarded as sufficiently nutritive for bacterial growth to occur, they were merely inoculated with culture and incubated, and portions withdrawn and plated at intervals.

A second but simpler method for activity tests consisted in adding the test material to Petri dishes containing 0.5 cc. of defibrinated horse blood and pouring plates with melted agar inoculated with 16 hour broth cultures of the scarlet fever streptococcus. After 48 hours incubation the longest diameters of the hemolytic zones about several deep colonies were measured. An ocular micrometer and a mechanical stage micrometer were used with a 32 mm. objective.<sup>3</sup> It will be shown that such measurements offer a method of judging inhibitory activity. Data of this nature are given in Tables III A, IV A, and IV B. In each case a measurement indicates an average size of five hemolytic zones. These did not differ more than 0.1 mm. from the average. If the control has a size  $M_o$  and another measured zone has a size  $M$ , then  $100 M/M_o$  is the "per cent of control." In the tables we have given values of "100 minus per cent of control:"

$$\text{"100 minus per cent of control"} = 100 - 100 \frac{M}{M_o} = 100 \left( 1 - \frac{M}{M_o} \right)$$

This gives a value which increases with the lactenic activity. Boiled whey has a value of zero and whey (from milk heated at 58°C. for 20 minutes) has a value of 100.

To obtain the activity of a preparation, its data are plotted as in Figs. 1, 2, or 3 together with the data of untreated whey. The length of a horizontal line connecting the two curves gives the logarithm of the relative activity.

*The effect of pH on lactenin* is shown in Experiment 1.

*Experiment 1.*—To 10 cc. portions of whey various quantities of  $\text{N HCl}$  and  $\text{N NaOH}$  were added (as shown in Table I). The tubes were then incubated 1½ hours and the reaction adjusted to the original pH. Each lot was then distributed in small tubes and refrigerated overnight. The next morning the tubes were inoculated with the 16 hour broth culture and the plates prepared before incubation and at intervals thereafter. All plate cultures were incubated 24 hours when the colonies were counted.

The whey retained its activity over a relatively narrow range of acidity and alkalinity. Thus when the acidity was brought to pH

<sup>3</sup> We are indebted to Dr. M. Kunitz of The Rockefeller Institute for Medical Research for helpful suggestions in regard to this method of measurement.

3.93, a point, it should be said, below that associated with the bacterial souring of milk, lactenin was not appreciably inactivated. A lower pH resulted in injury, and at pH values still lower than those shown in Table I it was completely inactivated. Similarly, although a reaction of pH 10.24 failed to decrease the inhibitory activity, when stronger alkali was added the inhibitory property was lost.

TABLE I

*The Effect of Acidity and Alkalinity on the Lactenic Activity of Whey*

Indicated amounts of  $M$  HCl or  $M$  NaOH were added to 10 cc. samples of whey, which were then allowed to stand 1.5 hours, neutralized and tested.

Volume HCl	pH before neutralization	Streptococci per cc. after					
		0 hrs.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.
cc.							
0	6.8	384	422	320	320	2	0
0.15	4.9	371	282	323	307	5	0
0.2	4.57	323	307	371	294	8	0
0.25	4.26	409	358	320	371	13	0
0.3	3.93	345	512	397	435	17	0
0.4	3.26	422	550	473	6,528	$\infty$	$\infty$
Control*		499	5,504	86,400	$\infty$	$\infty$	
Volume NaOH							
0	6.54	704	716	589	412	2	0
0.15	9.5	742	537	461	448	0	0
0.2	9.67	691	627	525	576	0	0
0.25	10.0	665	665	768	576	24	0
0.3	10.33	742	614	627	576	0	0
0.4	10.58	768	883	934	806	25	1
Control*		1,024	15,550	43,000	$\infty$	$\infty$	

\* For a control, portions from all tubes were combined and boiled.

*The Effect of Dialysis.*—As stated in the introduction, whey contains considerable sugar and salts in addition to 0.4–0.5 per cent protein. It will be shown in Experiment 2 that the sugar and salts may be completely removed by dialysis, leaving the protein in the collodion bags together with essentially all the lactenin.

*Experiment 2.*—Whey from the mixed milk of a number of cows was prepared as usual. Measured portions were placed in tightly stoppered sterile collodion

sacs and dialyzed for 24 hours with flowing distilled water at room temperature in an apparatus which has been previously described.<sup>4</sup> The tubes were mechanically agitated throughout this period. Ordinary tests failed to show the presence of salts or carbohydrates in the dialyzed whey; a portion of the protein was precipitated but considerable still remained in solution. During dialysis the acidity was increased from pH 6.6 to pH 6.2. As a control a portion of the original whey was kept in the room during the dialysis. Both lots were then filtered through Grade V Berkefeld filter and tested for their inhibitory property.

That considerable activity was still retained after dialysis is brought out in Table II.

TABLE II  
*Effect of Dialysis on the Lactenic Activity of Whey*

Product	Number of colonies per cc. after					
	0 hrs.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.
Whey.....	802	488	192	77	38	896
Boiled whey.....	1,600	23,360	∞	∞	∞	
Dialyzed whey.....	640	960	2,988	1,408	6,848	1,024
Boiled and dialyzed whey.....	1,088	4,544	14,400	∞	∞	∞

When the effects were measured by adding various quantities of both whey and the dialyzed product to Petri dishes little difference in inhibitory quality could be shown. When 1 cc. portions of each product were added growth was completely inhibited. When smaller quantities were used the colonies became progressively larger and the hemolytic zones more fully developed. During dialysis a small precipitate was formed which was slightly inhibitory.

*Proteolysis, Dialysis and Concentration.*—The solution left in the collodion bags was shown to be free from salts and sugar but contained the lactenin together with 0.4–0.5 per cent protein. Attempts to concentrate this dialyzed whey by the usual means failed, and attempts to fractionate the protein by precipitation with alcohol, ammonium sulfate, etc., resulted in inactivation.

It was now found that a part of the remaining protein could be removed by the action of trypsin followed by dialysis. In Experiment

<sup>4</sup> Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1928, 11, 641.

3 whey was treated in this manner. The dialysis was performed in the concentrating dialyzer described in the preceding article,<sup>5</sup> and was concentrated nearly to dryness. This concentrated solution, when diluted back to its original volume, contained only 0.12 per cent protein but was essentially as active as the original whey, and when tested in the concentrated form it completely prevented growth of the scarlet fever streptococcus.

TABLE III A

*The Lactenic Activity of Whey and of the Concentrated Products of Whey as Measured by the Hemolytic Zones of the Scarlet Fever Streptococcus*

Dimensions represent longest diameter of the hemolytic zones in mm. Each value is the average of five readings which differ by less than 0.1 mm. from the average.

Volume per plate culture.....	1.00 cc.	0.50 cc.	0.25 cc.	0.125 cc.	0.063 cc.	0.031 cc.	Relative activity	
							In conc. used	In original volume
Boiled whey (control) average (mm.)		2.4					0	0
Whey average (mm.)	0.52	0.94	1.36	2.12				
100—% of control	78	61	43	9			1.0	1.0
Concentrated digested dialyzed whey (1/20 of original volume)								
average (mm.)			0*	0*	0*	0.52		
100—% of control			100	100	100	78	32	1.6
Concentrated digested dialyzed whey rediluted to original volume								
average (mm.)	0.5	1.16	1.6	2.1				
100—% of control	79	52	33	9			0.9	0.9
Residue from 1 cc. digested dialyzed concentrate in 4 cc. NaCl + NaHCO <sub>3</sub> (1/5 of original volume)								
average (mm.)	0.98	2.2						
100—% of control	59	8					0.3	0.06

\* Zero size means not visible by 40 diameters magnification.

<sup>5</sup> Simms, H. S., *J. Exp. Med.*, 1930, 51, 319.

*Experiment 3.*—A trypsin solution was made by suspending 5 grams of Fairchild's trypsin in 100 cc. of sterile NaCl solution and passing the centrifuged supernatant fluid through a Berkefeld V candle. Whey, prepared from fresh milk which had been heated at 58°C. for 20 minutes, was treated with this trypsin solution in the proportion of 1 cc. of trypsin solution for each 20 cc. of whey. The whey-trypsin mixture was incubated 2 hours and placed in sterile collodion sacs, then dialyzed and concentrated in the apparatus described in the preceding article.<sup>5</sup> After 4 hours the dialyzed material was withdrawn from the sacs, pooled and measured. It was found that the digested material had been concentrated to 1/20 of its original volume.<sup>6</sup> It was chilled overnight in the refrigerator and a slight precipitate was centrifuged off.

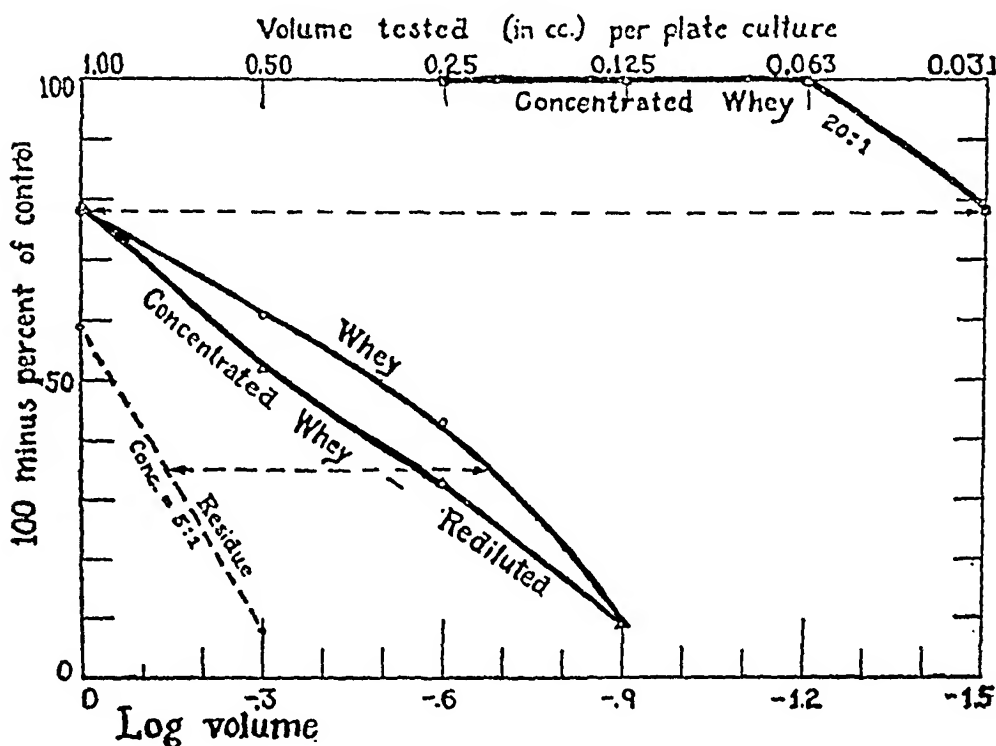


FIG. 1. The lactenic activity of whey and the concentrated products of hydrolyzed whey, as measured by the hemolytic zones of the scarlet fever streptococcus. The length of the horizontal dotted lines measure the logarithms of the relative activities.

<sup>6</sup> Concentration may be continued to complete dryness by this method, but it is best to remove the material when about 3 cc. is left in each bag. This avoids unnecessary loss.

A portion of the original whey which had been refrigerated during this period was tested as a control. Sterile salt solution was added to the control whey, in a volume proportionate to the trypsin used in the digested whey.

TABLE III B

*The Effect of Concentrated Dialyzed Digested Whey on the Multiplication of the Scarlet Fever Streptococcus*

Substance	Number of colonies per cc. after					
	0 hrs.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.
Whey.....	205	230	264	62		0
9.5 cc. boiled whey, + 0.5 cc. concentrated dialyzed digested whey.....	230	264	230	1,280	0	0
9 cc. boiled whey, + 1 cc. concentrate.....	230	264	51	26	0	0
9 cc. boiled whey, + 1 cc. concentrate boiled 5 minutes.....	294	2,816	17,280	∞	∞	
Boiled whey.....	320	4,548	∞	∞	∞	

*Part A. Testing Materials.*—Whey, concentrated material, and the residue centrifuged from the concentrated material were tested as to their effect on the hemolytic activity of the scarlet fever streptococcus. The residue was tested when dissolved in NaCl solution and a little  $\text{NaHCO}_3$ . The concentrated digested whey was tested both as such and also when diluted to its original volume.

The measurements given in Table III A are those of the longest diameter of the zone of hemolysis about the deep colonies of the scarlet fever streptococcus, as described above. The data are also plotted in Fig. 1.

*Part B. The Effect of the Addition of Concentrated Material to Boiled Whey.*—When the concentrated digested material is added to boiled whey in amounts sufficient to dilute it to its original concentration, or twice its original concentration, the inoculated streptococci fail to multiply and eventually die (as shown in Table III B). The boiling of whey destroys its lactenic activity so that growth begins soon after inoculation, but addition of the concentrated material prevents multiplication.

*The Preparation of Active Dried Material.*—The following experiment shows that the material of Experiment 3 may be completely desiccated with but slight decrease in activity. The material when partially dried is sometimes clear yellowish and vitreous, like dried varnish, but when completely dried is easily crumbled to a fine powder.

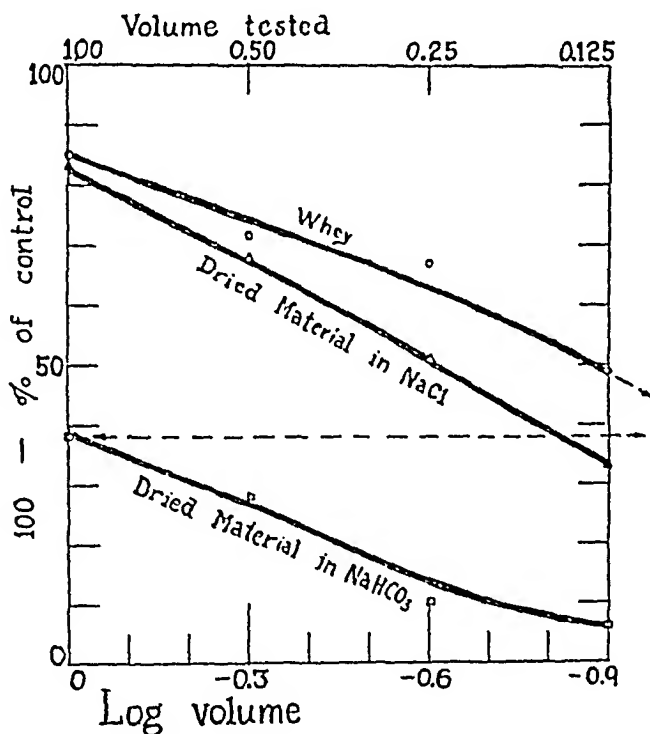


FIG. 2. The lactenic activity of dried dialyzed hydrolyzed whey when diluted to its original volume.

*Experiment 4.*—A portion of the dialyzed digested material which had been concentrated in Experiment 3 to 1/20 of its original volume was dried in high vacuum over  $\text{CaCl}_2$ . After 36 hours the dried material was divided into two portions and NaCl solution was added to one in an amount equivalent to the original volume of whey from which it had been obtained. The remainder was taken up in a proportional amount of NaCl solution containing N/10  $\text{NaHCO}_3$ . After thorough mixing both lots were stored in the refrigerator for 18 hours and then tested as in the method described. The measurements of the longest dimension of the hemolytic area are given in Table IV A and plotted in Fig. 2.

There is no indication that the substance has been materially injured by drying and the other steps in the process, provided that it is

TABLE IV A

*Lactenic Activity of Dried Dialyzed Whey When Diluted to Its Original Volume*  
(From longest diameters of hemolytic zones; see Table IIIA)

Volume per plate culture.....	1.00 cc.	0.50 cc.	0.25 cc.	0.13 cc.	Relative activity original volume
Control average (mm.)	2.76				
Whey* average (mm.)	0.46	0.76	0.9	1.4	
100—% of control	85	72	67	49	1.0
Dried material suspended in NaCl solution (pH 6.0)					
average (mm.)	0.46	0.88	1.36	1.86	
100—% of control	83	68	51	33	0.7
Dried material suspended in 0.1 M NaHCO <sub>3</sub> (pH 9.0)					
average (mm.)	1.7	2.0	2.3	2.6	
100—% of control	38	28	10	6	0.1

TABLE IV B

*Lactenic Activity of Dried Material Kept 3 Months and Diluted as Indicated*  
(From longest diameters of hemolytic zones)

Volume per plate culture.....	1.00 cc.	0.50 cc.	0.25 cc.	0.13 cc.	Relative activity in original volume
Control average (mm.)	2.64				
Whey* average (mm.)	0.42	0.62	0.82	1.14	
100—% of control	84	76	69	57	
Dried material after 3 months, diluted to original volume					
average (mm.)	0.54	1.12	1.96	2.14	
100—% of control	80	58	26	20	(0.7)*
Residue from above solution suspended in 1/3 of original volume					
average (mm.)	0.44	0.79	1.5	2.34	
100—% of control	83	70	43	10	(0.3)*

\* The whey in this experiment is fresh and therefore cannot be accurately compared with the 3 months old preparation. The fresh whey is included in this experiment as a control on the culture of streptococcus. The values of relative activity in the last column are therefore not quantitatively correct.



resuspended in NaCl solution. If, however, sufficient sodium bicarbonate is added to the salt solution so that the final reaction is pH 9.0, considerable destruction takes place during overnight refrigeration.

*The Effect of Time.*—Dried material prepared in the same manner and stored 3 months in the refrigerator and in the room, still retained much of its activity, as shown in Table IV B and Fig. 3.

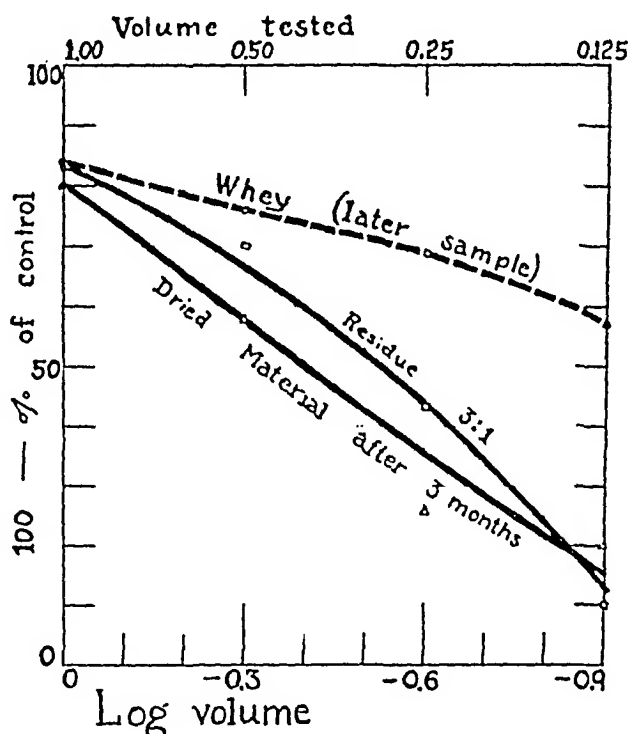


FIG. 3. The lactenic activity of the dried material after standing 3 months

*Highly Active Material.*—When sufficient salt solution was added to the dried material to bring it to the volume of original whey a portion failed to dissolve. This fraction from 12 cc. of whey was separated by centrifugation and suspended in 3 cc. of NaCl and 1 cc. of N/10 NaHCO<sub>3</sub> and tested. The results are included in Table IV and Fig. 3. It was found to have nearly one-half the activity of the dissolved portion. But it represents only one-eighth the dried weight. Hence this residue is 3 or 4 times as active as the dissolved portion on the basis of dry weight, or *about 200 times as active as skimmed milk*, on the same basis.

## DISCUSSION

The experiments give us some information concerning the properties of lactenin. It is stable for  $1\frac{1}{2}$  hours at pH values ranging from 4 to 10. If the time of exposure be lengthened the material must be kept even closer to the neutral point. We have shown that lactenin is not associated with salts and carbohydrates but can be readily separated from them by dialysis. Lactenin appears to be associated with the proteins of whey, since agents which precipitate protein from whey also remove the lactenin, although lactenin is not removed with casein during rennet coagulation. Furthermore lactenin, like proteins, fails to pass through permeable collodion membranes. It and some of the whey proteins resist the digestive activity of trypsin, but the split products of the proteins hydrolyzed by trypsin are removed by dialysis without impairing lactenic activity. We have no evidence that lactenin is itself a protein.

TABLE V

*Activity of Lactenin Material on a Fat-Free Dry Weight Basis*  
(Skimmed milk is taken as unity)

Material	Per cent solids	Relative activity per gram dry weight
Whole milk	13.6	1.0
(Fat removed by centrifuging)	<u>-4.0**</u>	
Skimmed milk	9.6	1.0
(Casein removed with rennet)	<u>-3.1</u>	
Whey	6.5*	1.5
(Salts and sugars dialyzed out)	<u>-6.1</u>	
Dialyzed whey	0.41*	23
(Proteins hydrolyzed and removed by dialysis)	-0.29	
(Protein added with trypsin)	<u>+0.003</u>	
Dialyzed hydrolyzed whey	0.123*	70
(Redissolved)	<u>-0.109</u>	56
Residue on redissolving	0.014*	205

\* Dry weights determined.

\*\* Normal value assumed.

In Table V will be found the dry weights of various lactenin materials. It will be seen that the dialyzed hydrolyzed whey contains

only 1.3 per cent of the original fat-free solids of milk. The tests indicate that this contains at least 90 per cent of the original activity of milk.<sup>7</sup> Hence it is 70 times as active as skimmed milk on a dry weight basis.

The material which has been digested, dialyzed and concentrated may be dried and stored for some time without appreciable loss of activity. When this material is dissolved it is about 56 times as active as the original milk, on a dry weight basis. A portion of the material fails to dissolve in physiological NaCl solution. This fraction is 205 times as active as the original milk, on a dry weight basis. The chemical nature of this very active material will be discussed in a future publication.

The method of measuring the size of hemolytic zones proved to be a reliable index of lactenic activity of substances which are not highly nutritive. It is possible to measure the effect of as little as 0.13 cc. of whey or other active products when mixed with 12.5 cc. of nutritive medium (veal infusion agar + horse blood) upon the development of the colonies and hemolytic zones of the scarlet fever streptococcus. With whey this means that its activity is shown in a final dilution of 1:100. The residue from the dried material is active with 0.005 mg. per cc. of plate culture. The relative activity of different materials is obtained by the horizontal distance between curves of data plotted as in Figs. 1 to 3.

#### SUMMARY

The bacterial growth inhibitory substance found in milk is called *lactenin* in this paper. It is stable for  $1\frac{1}{2}$  hours at pH 4 and at pH 10 and for longer periods in neutral solution. It is not associated with salts and carbohydrates and may be separated from them by dialysis.

Lactenin is removed by agents which precipitate the proteins of whey. Part of these proteins may be hydrolyzed by tryptic digestion and the resulting split products, together with the salts and sugar, may then be removed by dialysis without appreciable loss of lactenic activity.

<sup>7</sup> If we allow a 10 per cent loss of material in removing it from the bags, there is no appreciable loss in activity.

This dialysis may be performed in a concentrating dialyzer, under sterile conditions and at low temperature, thus reducing the solution to small volume. The material may then be completely desiccated and kept 3 months with practically no loss of activity. The residue, on treating this dried material with salt solution, is 200 times as active as the original milk, on a dry weight basis.

The size of hemolytic zones of the scarlet fever streptococcus grown on a medium containing lactenin is found to furnish a simple and reliable measure of lactenic activity.



## CHEMOTHERAPEUTIC EQUILIBRIA

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The statement has been made (1), with reference to the apparent fruitlessness of the search for specific chemotherapeutic agents, that "the real difficulty lies in the necessarily opportunistic experimental method, and the lack of a rational scientific means of approach." The considerations presented in the present paper are sufficiently general to be valid regardless of the mechanism of chemotherapeutic action which may be eventually discovered; and it is hoped that they will suggest a tentative rational approach to such a study.

### *Bacteriochemical Equilibria*

A large amount of work done *in vitro* has indicated that the reaction between the bacterial cell and some, at least, of the common chemotherapeutic agents is chemical in nature and indicates a behavior which can be predicted by the law of mass action (2).

The bacterium is pictured as an equilibrated amphoteric system of more than one component. Such systems have been shown to behave in many respects as simple ampholytes (3).

The belief that the reaction between the organism and the agents studied is chemical is founded on extensive studies of staining behavior, bacteriostatic behavior, and the effect of oxidizing agents and decolorizing agents on the former. The following series of equilibria may be thought of as establishing themselves in a system of bacteria and basic agent. For considerations of staining actions this agent will be a basic dye; for bacteriostatic considerations it may or may not be a dye. We will indicate the fact that the agent is basic by the type formula DOH, and that the bacterium is amphoteric by the formula HBOH.

1.  $\text{DOH} \rightleftharpoons \text{D}^+ + \text{OH}^-$
2. a)  $\text{HBOH} \rightleftharpoons \text{H}^+ + \text{BOH}^-$   
b)  $\text{HBOH} \rightleftharpoons \text{HB}^+ + \text{OH}^-$
3.  $\text{D}^+ + \text{BOH}^- \rightleftharpoons \text{DBOH}$
4.  $\text{DBOH} + \text{HOH} \rightleftharpoons \text{HBOH} + \text{D}^+ + \text{OH}^-$

Maximum retention of stain, or maximum bacteriostatic effect, will be expected under conditions favoring maximum formation of the unionized dye-bacterial compound, DBOH (Equation 3). The longer arrows in the above set of equations indicate the directions in which the equilibria would shift due to an increase in alkalinity.\*

For staining reactions we have the following predictions and verifications:

1. Increase in pH should increase the retaining power for dye. This fact is now generally recognized. At sufficiently high pH values even Gram negative organisms may appear to be Gram positive (2c).

2. The acidic strength of the organism should be a factor. Actually, determinations of the isoelectric ranges of a number of organisms showed that these ranges in the case of Gram positive organisms occurred at a significantly higher acidity than those characteristic of the Gram negative organisms as a class (2f) (4).

3. Any process which tends to increase the acidic strength of the same organism should increase its inherent retaining power for dye. Actually it has been shown that by oxidation the retaining power of any organism for basic dye is increased. By sufficiently vigorous oxidation any Gram negative organism can be made to appear Gram positive. Subsequent reduction tends to reverse this effect (2c).

4. If these reactions represent the partial attainment of an equilibrium condition, they should be easily reversible. Actually this is demonstrated by the ease of decolorizing any non-capsulated organism with an acid decolorizer.

5. If the type of bond between organism and dye is chemical, then the general nature of the decolorizer used should determine its functioning as a decolorizer apart from the mere solubility of the dye in it. In this connection the authors have shown (2j) (2k) that basic decolorizers show abnormally high decolorizing power toward smears stained with acid dyes, and acid decolorizers show the same abnormal behavior toward smears stained with basic dye. It should be borne in mind that this effect is independent of any pH effect on decolorization and depends only on the chemical nature of the decolorizers. For example aniline would be a representative basic decolorizer while aldehydes would be acidic in nature.

In the case of acid dyes what may be thought of as a reverse behavior to that toward basic dyes has been in every case found to exist. Such could be predicted from an analogous set of equations.

Analogously we would have for bacteriostatic reactions, the following predictions and verifications:

1. Increase in alkalinity, which shifts the equilibria toward the formation of com-

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\* Equation 4 takes account of the fact that the effect of increasing alkalinity on the amphoteric cell is much greater than on the purely basic dye through the ordinary pH range where such studies are of importance, even though the respective ionization constants may not be so widely different. In this pH range the dyes, at least, would be almost wholly in the form of salts and thus, for comparison of effect only, the dye is represented as being highly ionized compared to the protein constituent.

pounds represented by the type formula DBOH in the equations, should increase the effective dilution of a basic bacteriostat. In the case of dyes this is, as is now well known, the case where such increase in alkalinity is not sufficient to precipitate out the agent or to alter its chemical nature (2g) (2h).

2. These bacteriostatic effects should also be reversible. That is, as an example, a concentration of a dye which would just inhibit growth at a certain pH should fail to inhibit it at a lower pH. This has been definitely shown by the authors (2g). Such a reversal can also be brought about in other ways. Any method of dissociating the molecule represented by DBOH by removing the basic  $D^+$  ion will be effective. This is shown by the work of Englehardt (5), who removed mercuric ion by sulfide precipitation, and found that staphylococci which had been treated for 72 hours in a 1% bichloride solution would, after the removal of the mercuric ion, grow out. Similarly apropos in this connection are the results obtained by Churchman (6) on injection of stained *Bacillus anthracis*, which were found to remain apparently innocuous for periods from 10 to 20 times longer than those required by unstained organisms to produce death, but which finally suddenly revived with fatal results. Here, of course, we cannot put our finger on the chemical mechanism by which the positive dye ion was removed from action.

3. We should expect increase in basic strength of dye to increase its effectiveness. As the authors have pointed out (2e), all available data indicate such to be the case, but such data are meager. This factor cannot be generally treated because different classes of agents show effects which are of an entirely different order of magnitude. Also through the pH range usually studied most of them will be in salt form and but slightly hydrolyzed.

4. The presence of protein or other matter of a similar nature which chemically resembles bacteria should decrease the effectiveness of any agent by binding a portion of it in an ineffective combination. This has been shown to be so by Graham-Smith (7), Winslow and Dolloff (8), Wels (9), and others.

5. If mass action tends to govern bacteriostatic effects, the amount of bacterial inoculum should be a factor. That this is so has been qualitatively demonstrated by many, and semi-quantitatively shown by Gay and Beckwith (10), Browning and Gulbransen (11) and Graham-Smith (7), among others. While this last mentioned phenomenon has received other explanations, it is at least completely in harmony with the point of view outlined above.

6. Finally we may expect the acidic strength of the bacteria to be a factor. The well known fact that basic dyes are more effective against Gram positive than against Gram negative organisms, coupled with our findings on the isoelectric ranges of these organisms showing the Gram positive ones to be more strongly acidic, constitutes the experimental verification of this prediction.

The probability of the general correctness of the above outlined point of view is strengthened by the analogous behavior of organisms toward acidic substances, which behavior can also be successfully predicted from an analogous set of equations, as well as by the general similarity of all such behavior to the simple protein chemistry.



*Stoichiometrical Relationships*

Whatever be the mechanism of internal therapy, there seems to be evidence *in vitro* of some action between agents and organism which follows ordinary stoichiometrical laws. It is therefore of interest and importance, in the first place, to inquire whether the quantities of therapeutic agent which have been suggested from time to time are stoichiometrically sufficient. Recently the dyes gentian violet and mercurochrome have been used to a considerable extent in blood therapy. Take the former as an example.

A concentration of 1:10,000 in the blood stream has been found safe and has been used. Taking the equivalent weight of the dye as about 400, we have, at this

TABLE I

*Showing the Effect of Blood Constituents on the Effective Concentration of Dye against Bacteria in the Blood*

Equiv. dye ion orig.	Approx. equiv. protein anion	Approx. ion- ization const. for dye-protein salt*	Equiv. dye ion. final	Equiv. bac- terial protein	Approx. ratio Dye equiv. Bact. equiv.
1	20 (plasma)	0.0005	0.01	0.00004	250.
1	35 (whole blood)	0.0005	0.006	0.00004	150.

\* This magnitude is obtained from experiments of the type described in section on "Abnormal ionization equilibria."

concentration, one equivalent of dye to about 4,000,000 parts of blood. In the blood we have, besides the bacteria which are being combated, the blood proteins to consider, especially those of the plasma which, at the pH of blood, are on the alkaline side of their isoelectric points. These proteins are in large excess compared with the bacteria. If we assume that they have characteristics not too dissimilar from those simple vegetable proteins so exhaustively investigated by Hoffman and Gortner (12) we can get a fair idea of the order of magnitude of the concentration of protein anion. In Table I are recorded results of such calculations. We have assumed the composition of the blood as given by Mathews (13). A preliminary determination of the ionization constant of the dye base of gentian violet indicated that, since it is added as the chloride, the dye will be present practically entirely as cation until bound by bacteria or some component of the blood. The number of equivalents of fibrinogen can be assumed by reading directly from the curves of Hoffman and Gortner for fibrin; while the number of equivalents of the other proteins are assumed to be about the same as they would be if they consisted of the

prolamines studied by these workers, twelve of which showed almost identical behavior at blood pH. The various quantities in Table I are calculated on the basis of one equivalent of dye ion, i.e. for 4,000,000 parts of blood.

The bacterial equivalents are calculated on the assumption of a count of 10,000 per cc. (2e). The final ratios, then, represent lower limiting conditions. Work on the staining behavior of blood cells, both sheep and human (15) indicates that the cells as a whole have an isoelectric point at a pH of about 6 to 6.4 so that at blood pH they are just beginning to show an appreciable affinity for basic dye. It is therefore more than probable that they do not affect the effective dye concentration to nearly the extent assumed in the values given in Table I. Moreover a bacterial count of 10,000 per cc. seems to be also a fairly limiting case. Under actual conditions the effective dye-bacterial ratio would probably be nearer many hundred than the values given.

These values indicate that, even in blood where it might be expected that dye would be completely combined with blood proteins which are somewhat in excess, a total dye concentration found clinically safe to use, 1:10,000, may be expected to furnish a bacteriostatic value corresponding to an *in vitro* concentration of 1:1,000,000 or 1:2,000,000 (i.e. 0.01 to 0.006 parts in 10,000 as in column 4 of Table I). These latter concentrations are not only in large stoichiometric excess over bacterial equivalents but are concentrations found bacteriostatic *in vitro* for many strains, especially of Gram positive organisms.

Recently some work reported by Hirschfelder and Wright (14) indicates that a concentration of crystal violet 1:20,000 in the presence of 1% albumin showed the same antiseptic power as a 1:35,000 solution in the absence of protein. No mention is made of buffering the system though the results were obtained by a process in which CO<sub>2</sub> was liberated. This, in an unbuffered system, might significantly affect the pH, which has been shown (2g) to greatly influence the effectiveness of dyes, and one cannot judge critically the conclusions of the authors that even adsorbed dye has some antiseptic value. This conclusion was reached on the basis of calculations from adsorption data which indicated that this 1:20,000 dye solution should have had the same antiseptic power in 1% albumin as a 1:125,000 solution in water. Details of measuring adsorption are not given so that one cannot judge the applicability of these results to blood conditions, but the work would tend to confirm the conclusions reached from the above calculations.

### *Time of Establishing Dye-Protein Equilibrium*

A puzzling feature regarding the action of therapeutic agents, especially in the blood, which has often been brought up, is the apparent rapid disappearance of the agent. In this connection the results of two experiments are presented.

Table II embodies the results of some time experiments on the establishment of practical equilibrium between finely cut dry gelatin slabs and solutions of gentian

violet. Naturally the time values may be in error by 50% or more on such short times, but the results are suggestive. In obtaining these results definite amounts of gelatin were shaken vigorously with definite volumes of gentian violet solution of known concentration and controlled alkalinity, for definite periods of time. A portion of the dye was by this means bound by the gelatin and removed from

TABLE II

*Showing the Approximate Length of Time Required for Equilibrium between Gentian Violet and Gelatin*

cc. Dye soln.	Grams gelatin	cc. N/2 NaOH added	Time shaken (sec.)	Orig. dye concn. p.p.m.	Final dye concn. p.p.m.	Final dye concn. p.p.m. (blank)
10	0.08	—	60	10	8.0	9.0
10	0.08	0.20	10	10	5.0	
10	0.08	0.35	10	10	2.0	7.5
10	0.10	0.20	10	10	5.0	9.5
	(same tube)		25		3.0	
			40		3.0	
			60		3.0	8.5

TABLE IIa

10	0.10	—	20	10	7.0	
10	0.10	0.04	12	10	6.0	
10	0.10	0.20	10	10	4.0	9.0
10	0.10	0.40	10	10	3.5	9.0
10	0.10	—	20	5	3.5	
10	0.10	0.04	12	5	3.0	
10	0.10	0.20	10	5	1.5	
10	0.10	0.40	10	5	1.0	4.5

(Longer shaking in these latter cases made no difference in the results.)

Results in Table II were obtained using a solution of gentian violet in water; those in Table IIa were obtained using a solution of the dye in M/20 disodium phosphate.

solution, and the intensity of the color remaining in the supernatant liquid was compared with standard solutions of the dye.

This experiment indicates that, if the above pictured mechanism of bacteriostasis is true, the time required for a bacteriostat to become effective is conditioned largely by speed of mixing with an infected fluid and perhaps of penetration into organisms. The actual bacteriostatic equilibrium seems to establish itself very rapidly when mutual contact of dye with the particular component with which it may combine is obtained.

This simple experiment indicates that, when there are no disturbing factors and when agitation is vigorous, a primary equilibrium is very rapidly reached between protein and dye. Although, in the case of blood therapy, the method of administration of the agent would seem to lead to a rather rapid and thorough mixing, one may expect in such a medium, where blood proteins are so enormously in excess of bacterial protein, that the time required by the agent for action may be considerably increased.

It should be pointed out that the time required for the establishment of this type of equilibrium should not be confused with the times, reported through the literature, required for "killing" an organism. An agent in the blood may be effectively holding in check the normal development of an organism, yet when a sample of this same blood is plated out it may appear by no means sterile. This may be due as we have seen above to an alteration of any of the factors which have been shown to reverse a bacteriostatic equilibrium.

#### *Alteration of Therapeutic Agent in the Blood*

The other consideration we wish to present in connection with the apparent rapid disappearance of such an agent from the blood is that this apparent disappearance may not mean that the blood no longer retains some effect. It seems to be necessary for certain agents, notably certain pentavalent arsenic preparations, to be reduced by the body tissues to trivalent arsenic before they produce the desired effect. It was on such a basis that Ehrlich accounted for the clinical trypanocidal effectiveness of atoxyl, which, *in vitro*, is ineffective against these organisms even at a concentration of 5 per cent. On the other hand the trivalent arsenic in the form of the oxide is immediately trypanocidal at a concentration of 1:100,000.

Gentian violet is rather easily decolorized with either nascent hydrogen or hydrogen peroxide. The following experiments were performed.

Solutions of the colorless products were prepared using both methods. For the first method zinc and hydrochloric acid were employed, and the bulk of the zinc was removed by precipitation with ammonia. The resulting solution was tested for bacteriostatic action. Controls of ordinary gentian violet as well as of the zinc salt, prepared from zinc and acid as in the reduction mixture, were run. The bac-

teriostatic activity of a dye solution decolorized by hydrogen peroxide and in which the excess peroxide had been decomposed was also tested. Results are presented in Table III. They are, of course, preliminary in nature, but they seem to have a significance in themselves aside from their bearing on a rational picturization of a mechanism for blood therapy, especially in the case of agents which seem to rapidly disappear. It may be pointed out that reduction by zinc and acid does not

TABLE IIIa  
*Showing the Bacteriostatic Effect of Decolorized Gentian Violet*

Organism	pH	Zn soln. control			G. V. red. by nascent hydrogen sample # 1			G. V. red. by nascent hydrogen sample # 2			Normal G. V. control	Dilution of agent
<i>B. coli</i> ..... Strain 1	5.5	+	+	+	-	-	-	-	-	-	-	1:20,000
	6.5	+	+	+	-	-	-	-	-	-	-	1:20,000
<i>B. coli</i> ..... Strain 2	5.5	+	+	+	-	-	-	-	-	-	-	1:20,000
	6.5	+	+	+	-	-	-	-	-	-	-	1:20,000

TABLE IIIb

Organism	Dilution of agent	pH	G. V. red. by peroxide		G. V. red. by nascent hydrogen		Normal G. V. control
<i>B. coli</i> .....	1:10,000	5.5 to 6.0	-	-	-	-	- -
<i>B. coli</i> .....	1:20,000	5.5 to 6.0	-	+	+	+	- +

TABLE IIIc

<i>B. coli</i> .....	1:10,000	5.5 to 6.0	-	-	-	-	- -
<i>B. coli</i> .....	1:20,000	5.5 to 6.0	-	-	-	-	- -

give the colorless product quite a fair chance in this test, since the gelatinous zinc hydroxide, which comes down in considerable quantities, is bound to carry down with it a considerable amount of active material which is thus removed.

The medium was ordinarily plain nutrient broth adjusted to a certain pH and containing a known amount of bacteriostat. In Table IIIa are given growth results in two samples of the dye decolorized with nascent hydrogen for 24, 48 and 72 hour incubation periods. (In this particular experiment the medium was 1%

lactose broth.) In Tables IIIb and IIIc results are given for growth in one sample of dye decolorized by nascent hydrogen and in one decolorized by hydrogen peroxide. Results are for 24 and 48 hour incubation periods. A slow precipitation of zinc hydroxide, brought about by pH adjustment, seemed to carry down nutrient material and, presumably, active agent as well. Therefore a trial was made on tubes in which the precipitation was hastened as much as possible and the precipitate centrifuged out. 24 and 48 hour growth results are given in Table IIIc, otherwise this table is similar to IIIb.

We are not, of course, suggesting that gentian violet is decolorized by these mechanisms in the blood, but it is of interest to note that disappearance of color in the case of such a reagent does not in itself mean disappearance of therapeutic effect.

At first sight these results may seem in disagreement with the statement of Dubos (16) who, from a study of the bacteriostatic effect of certain dyes which form reversible oxidation-reduction systems, finds that "the dyes are not toxic in the reduced form." His results are not comparable with those reported here, however, since he is working with reversible systems, systems which are reduced by the medium rapidly even in concentrations as high as about 1:5,000 and therefore systems which would tend to poise the medium at a certain oxidation potential. This poisoning effect he points out as a factor in the mechanism of dye bacteriostasis. There is no doubt in the minds of the present authors as to the validity of this claim where it can be shown that the bacteriostatic systems are effective as poisoning agents. In this paper bases for the claim of another factor are given. The relative importance of the two factors will depend on the particular systems. The most direct evidence of this other factor, which may be thought of as covalent salt formation between bacteriostatic agent and some constituent of the organism, will be found below.

### *Flocculation Equilibria*

It may seem at first sight a far cry from the above specifically chemical equilibria to the phenomenon of flocculation, with its possible relation to agglutination, which we usually consider as a surface phenomenon of physical nature. Results given in Table IV are typical. From such results as these it is indicated that flocculation behavior is influenced by essentially the same factors and in essentially the same way as is staining behavior.

Using care to differentiate between so-called acid agglutination and flocculation produced by the agent under observation, the following facts were ascertained:

TABLE IV

Showing the Effect of Dye Concentration and of pH on the Time of Flocculation of the Gram Negative *B. coli* and the Gram Positive *B. cereus* by Means of Basic and of Acid Dye

Floc. time (min.)	Basic fuchsin						Floc. time (min.)	Acid fuchsin								
	1	2	pH (approx.)		3	4		5	6	1	2	pH (approx.)		3	4	5
Organism— <i>B. coli</i>																
Dye concentration 1:200																
1	—	—	—	—	+	+	1	±	±	—	—	—	—	—	—	—
3	—	—	±	+	+	+	5	±	±	—	—	—	—	—	—	—
4	—	±	±	+	+	+	8	+	±	—	—	—	—	—	—	—
17	±	+	+	+	+	+	44	+	+	±	—	—	—	—	—	—
Dye concentration 1:250																
1	—	—	—	—	±	±	7	±	±	—	—	—	—	—	—	—
3	—	—	—	±	+	+	12	+	±	±	—	—	—	—	—	—
7	—	—	±	+	+	+										
22	±	+	+	+	+	+										
Organism— <i>B. cereus</i>																
Dye concentration 1:200																
0.5	—	—	—	—	+	+	2	±	±	—	—	—	—	—	—	—
2	—	—	+	+	+	+	11	+	+	±	—	—	—	—	—	—
3	—	±	+	+	+	+	19	+	+	±	—	—	—	—	—	—
4	±	+	+	+	+	+	60	+	+	+	±	—	—	—	—	—
Dye concentration 1:250																
1.0	—	—	—	—	±	+	1	±	±	—	—	—	—	—	—	—
1.5	—	—	—	±	+	+	7	+	±	—	—	—	—	—	—	—
2.0	—	—	±	+	+	+	17	+	±	±	—	—	—	—	—	—
2.5	—	±	+	+	+	+	60	+	+	±	—	—	—	—	—	—
6.0	+	+	+	+	+	+										

1. The basic dyes, gentian violet and fuchsin, are more effective in flocculating both the Gram negative *B. coli* and the Gram positive

*B. cereus* at higher than at lower pH values. That is, at constant dye concentration the flocculation is more rapid, while to attain a certain flocculation speed less dye is necessary, at high than at low pH values.

2. The reverse is true in the case of acid fuchsin. It should be pointed out in connection with material presented under "Abnormal ionization equilibria" that neither brucine nor nicotine cause flocculation at concentrations comparable to those of the dyes mentioned above. The experiments were made repeatedly, checking the macroscopic readings by microscopic examination.

If we picture the cell membrane, as suggested by Bayliss (17), as a variable structure in equilibrium with the changing states of the cell, the above results are easily brought into harmony with the general picture.

Any material within the cell which lowers the surface energy will accumulate at the surface. It has been shown (18) (19) (20) that alteration of the surface tension of media through wide ranges has no apparent effect on the viability of many bacteria. This means that the bacterial cell possesses a mechanism for adjusting its surface tension in response to changes in the surface tension of the medium so as to bring about a fairly constant interfacial tension between its surface and the medium. The obvious mechanism is a labile distribution equilibrium, between the surface and the interior of the cell, of some surface tension depressant such as a lipin or even a protein constituent. Addition of a reagent which possesses the power to bind such a substance will of course shift any surface equilibrium with the result that the interfacial tension between organism and medium may increase. The tendency therefore will be to decrease total surface and flocculation will result. Such binding agents are the dyes in the above experiments, their binding power having been previously shown to vary with pH in the manner necessary to explain the flocculation results.

It may be suggested that change in pH and addition of dyes may alter the surface tension of the medium in a way to cause flocculation. Measurements of surface tension of nutrient broth and of broth containing as high as 1 per cent dye and with pH varying between 2.5 and 8 gave a variation of only about 5 dynes, however, so that some other explanation must be sought than this.



*Abnormal Ionization Equilibria*

The question may well arise why, if we imagine the action of therapeutic agents to be pictured as taking place according to the set of equilibria formulated above, there is such a wide difference between the inhibiting dilutions of different groups of agents which seem to belong to the same general chemical type. More specifically the question may be put why such substances as the triphenylmethane dyes, for example, inhibit bacterial growth at effective concentrations enormously less than those at which other basic substances, whose basic strength may be as great or greater, are found to be effective. This question has led one of the authors to an investigation of equilibria of the type represented by equation number 3 above, namely the ionization of the compound represented by the formula  $\text{DBOH}$ . Obviously if this compound is highly ionized enormous excess quantities of  $\text{D}^+$  ion would be necessary for it to be formed in appreciable amount.

To determine whether different types of basic substances behave in a significantly different manner with regard to the ionization of their protein salts, conductivity measurements were made on solutions of certain bases alone, on a solution of protein alone, and on mixtures of the two. Results are given in Table V.

For the protein a 1% gelatin solution adjusted to a given pH by means of NaOH was used. For the bases, potassium, as the chloride, nicotine as the free base and gentian violet as the chloride were employed at concentrations equivalent to that of the gelatin taking its equivalent weight toward bases as 3300 (21).

In the cases of both nicotine and potassium the measured conductivity of the mixture agrees with that calculated on the assumption that none of the ionic species present tend to form undissociated molecules. In the case of the dye-protein system, however, there is a significant "loss of conductivity" when the two are mixed. A considerable portion of the dye or protein ion disappears, and this can be most easily pictured as being due to the fact that we are here dealing with a salt type of compound which is only slightly ionized, analogous in its behaviour to such inorganic salts as lead acetate, mercury salts, and others, which, compared with the general run of inorganic salts, are only slightly ionized.

The results of this experiment offer an explanation for the difference in flocculation behavior between the alkaloids and dyes noted in the above section. In the same way they offer an explanation for the

findings (22) that nicotine, even at a concentration of 1:2,000, does not seem to inhibit the growth of either Gram positive or Gram nega-

TABLE V

*Showing the Effect of Mixing Solutions of Protein Anion with Various Cations on the Conductivities of These Ions*

Solution	Temp.	pH	Conduc- tivity $\times 10^4$ (measured)	Conduc- tivity $\times 10^4$ (calculated)	% Differ- ence
A. Ionization of potassium proteinate					
KCl.....	23.5	unbuffered	108.6		
gelatin.....	23.5	7.65	38.4		
1:1 mixture.....	23.5	7.6	75.1	73.5	2.
B. Ionization of nicotine proteinate					
nicotine.....	24.9	9.5	13.5		
gelatin.....	24.9	9.4	39.0		
1:1 mixture.....	24.9	9.35	27.1	26.3	3.
C. Ionization of gentian violet proteinate					
gentian violet.....	23.6	7.25	27.0		
gelatin.....	23.6	7.3	38.4		
1:1 mixture.....	23.6	7.1	23.0	32.7	-30.
1 gel: 2 dye.....	23.6	7.15	18.5	34.6	-46.

The values in column 4, Table V, are the conductivities of the ions under consideration and not the total measured conductivities. KCl is corrected for the Cl ion, gelatin is corrected for the conductivity of the Na ion added in pH adjustment, etc. These corrections do not in any way affect the argument, though they do affect the magnitudes in the last column. If we take the total conductivities we find that for nicotine and potassium the observed conductivities for the mixture do not differ from the calculated values by more than a fraction of 1%. The decrease in the case of the dye in this way amounts to 13 to 18%. Since this decrease is not distributed among all of the ionic species present in the mixture but is practically confined to the ones discussed, a more definite idea of its magnitude can be had from the corrected data as given. The last system, namely gelatin and dye, has received fairly extensive study and results in detail are to be published elsewhere.

tive organisms. The dye in each case actually seems to bind, in unionized combination, some protein-like constituent of the bacterial

cell, in the one case shifting the surface equilibrium and causing flocculation, and in the other case interfering with normal cell development; whereas the alkaloid, not seeming to form such a type of linkage, is ineffective in both cases.

### *Ionic Displacement Reactions*

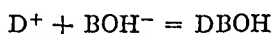
It may be suggested that the above conductivity results are explicable on the basis of the mutual flocculation of oppositely charged colloidal particles and has no connection with any type of salt formation. In answer to such a suggestion the following experimental results are presented.

TABLE VI

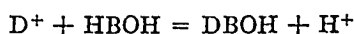
*Showing the Liberation of H-Ion When Gelatin Is Mixed with Dye Cation, and of OH-Ion When It Is Mixed with Dye Anion*

	Crystal violet		Acid fuchsin	
	pH	- Δ(pH)	pH	Δ(pH)
dye.....	3.92		3.13	
gelatin.....	3.91		3.13	
1:1 mixture.....	3.83	0.08	3.32	0.19
dye.....	4.64		3.75	
gelatin.....	4.64		3.75	
1:1 mixture.....	4.29	0.35	4.10	0.35
dye.....	5.64		4.60	
gelatin.....	5.64		4.62	
1:1 mixture.....	5.32	0.32	4.73	0.11

Consider an amphoteric substance represented by the type formula HBOH, which can exist in solution not only in the form of the neutral compound but also either as cation or anion depending on pH. Let it react with a basic dye ion and there are the following possibilities:



and



If these equations represent the types of reaction taking place between dye and protein, there ought to be conditions, if the compound DBOH is sufficiently stable, such that H-ion is liberated when dye ion and protein in unionized form are mixed.

In an analogous manner there ought to be conditions under which, when an acid dye ion is substituted for the basic dye ion, OH-ion should be liberated. This can be easily tested out by adjusting dye solutions and protein to the same pH and noting any change on mixing (23). A large number of such experiments have been carried out using various proteins and various dyes. Typical results are given in Table VI, using gelatin as a protein, crystal violet as a basic dye and acid fuchsin as an acid dye. The gelatin was a 1% solution and the crystal violet was used as a solution of 1.5 grams per liter. The other dye solution contained 3 grams per liter.

Here the basic dye causes a decrease in pH corresponding to a liberation of H-ion, and the acid dye causes the opposite effect.

Is it possible that types of therapeutic specificity will be found to be somehow connected with the property of forming such un-ionized compounds with a cell constituent? This is at least a point worthy of consideration.

#### SUMMARY

1. The general adequacy of the bacteriostatic mechanism for the action of dyes which postulates a mass law equilibrium between bacteriostat and organism, which latter is pictured chemically as an ampholyte, is discussed.

2. It is shown that, even in blood, where, with safe concentrations of dye, there seems to be a significant excess of protein over dye, the stoichiometric excess of dye required by the above mechanism is available.

3. Experiments are presented indicating that the time required for such a bacteriostat to act is very short, being probably conditioned largely by speed of mixing or of penetration.

4. Apparent disappearance of dye from blood stream need not mean that the blood has lost bacteriostatic value.

5. Data are presented indicating that the behavior of dyes in causing flocculation of organisms is affected by the same factors and in the same way as in inhibiting growth.

6. Direct evidence of ionic combination between dye ion and protein ion is presented by noting conductivity decrease when the two ions are mixed, and also noting the displacement of H-ion from un-ionized protein by dye cation, or of OH-ion by dye anion.

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# THE REFRACTORY PERIOD OF THE NORMALLY-BEATING DOG'S AURICLE; WITH A NOTE ON THE OCCURRENCE OF AURICULAR FIBRILLATION FOLLOWING A SINGLE STIMULUS

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## PLATE 8

(Received for publication, November 14, 1929)

In all previous determinations of the refractory period of the mammalian auricle two factors influencing the accuracy of the measurements obtained have been necessarily present as a part of the technique of the experimental procedure. In order to control the exact time in the cardiac cycle at which the interrupting stimulus is introduced it has hitherto been necessary to drive the heart by a series of break induction shocks at a rate more rapid than that of the spontaneous rhythm. The local stimulating effect of these repeated shocks upon the vagus endings of the myocardium has made accurate measurements possible only upon the atropinized heart, and in many instances has also led to an irregular failure of response at a level above the true refractory period. The occasional interruption of the rhythm by false responses, upsetting the essential order of at least several sequential cycles preceding that one during which the refractory period is determined, has also been unavoidable.

A second cause influencing the accuracy of the results previously obtained has arisen from the fact that it has been impossible to record the refractory period from the exact point of stimulation. In all such experimental observations there has necessarily been a small gap between the stimulating and the recording electrodes. It is a reasonable assumption that changes in this small area of musculature, as a result of the repeated induction shocks, may modify the measurement considered as the true refractory period of the auricular muscle. Even

when using the method first described by Lewis, Drury and Bulger (1) in which the shocks, which control the rhythmic beats of the heart, and those which test the refractory period, enter the muscle at precisely the same place, there is a gap of about 15 millimeters between the stimulating and the recording electrodes. This procedure has been necessary owing to the danger to the galvanometer string when the stimulating electrodes are placed too close to the leading-off point. As a result what has actually been measured has been the refractory period at the point of stimulation plus the interval required for the excitatory process to spread from this point to the recording electrodes. In the absence of any added experimental procedure, and under conditions in which both the refractory period and conductivity may be altered in the same sense, such results may be at least proportional to the true refractory period. The instant, however, that one attempts observations under the influence of pressure, cold, certain drugs or changes in the hydrogen-ion concentration of a perfusate, the possible effect of conduction with a decrement throughout this gap, can not be avoided (2). Under these circumstances, in some of which the refractory period may be shortened and conductivity remain unchanged or depressed, the error may be considerable.

By means of a balanced circuit with a double induction coil as the source of stimulus (as suggested by Bishop (3) and applied by Gilson (4)) it has been possible, in these experiments, to place the stimulating electrodes actually astride the proximal lead, thus avoiding any gap, and with no more disturbance of the galvanometric record than is shown in the accompanying curves (Fig. 1). Using a specially designed apparatus (See Appendix) the refractory period of the normally beating dog's auricle has been determined in the following manner.

The animals are anaesthetized with morphia (16 mg.) and urethane (1.0 gm. per kilo). The heart is exposed in situ, the pericardium opened and sewn back against the retracted ribs and a ligature is fastened to the tip of the right auricle drawing it toward the left chest wall. The action-current is led off from the auricular appendix by means of kaolin-paste copper-sulphate electrodes, amplified to 12 to 20 volts and applied to a light relay. Activation of this relay releases a pendulum, which, in turn, throws in a single break induction shock at a time interval determined by the position of a trip-switch along its arc, the normal excitatory process alone controlling the timing of the interrupting shock. The arm

bearing the trip-switch carries an accurately calibrated protractor scale etched upon its face. The local effect of a bombardment with induction shocks is thus avoided and there is the added advantage that the R-S interval may be accurately altered or exactly reproduced.

The threshold of current strength was first determined roughly by watching the heart and the galvanometer string, the point at which a response occurred being noted. The current strength was then doubled and a duplicate series of observations recorded from a time interval well above the observed refractory period to one well below. The refractory period was then always checked in the opposite direction; i.e. if the first determination was made with decreasing intervals, a second series was carried out with increasing intervals. It is of interest that the refractory periods so determined checked within a few thousandths of a second.

TABLE I

*Refractory Period of the Dog's Auricle. Spontaneous Rhythm. (R-S Intervals in Seconds)*

Dog No.....	15		22		17	
Auricular rate.....	170		150		185	
Refractory period	0.1408					
	0.1305	0.1385	0.1245		0.0978	0.1078
	0.1265 ↓	0.1316 ↑	0.1190 ↓	0.1153 ↑	0.0940 ↓	0.1008 ↑
	0.1250	0.1299	0.1159	0.1123	0.0895	0.0924
	0.1245	0.1275	0.1107	0.1100	0.0838	0.0878
	0.1210	0.1207	0.1095	0.1064	0.0781	0.0790
	0.1195 ↓	0.1144 ↑	0.1067 ↓	0.1037 ↑	0.0740 ↓	0.0774 ↑
	0.1160	0.1097	0.0978	0.1015	0.0685	0.0716
	0.1140	0.1021				0.0652

↓ with decreasing, and ↑ with increasing, intervals.

In a series of twenty experiments in which the refractory period was measured before stimulation of the vagus, the administration of atropine or adrenalin, values were obtained varying from between 0.0781 and 0.0858 to between 0.1392 and 0.1410. All measurements were made from the appearance of the excitatory process (R), to the onset of the interrupting stimulus (S)—the R-S interval.\* The close agreement of the two values obtained by the method outlined is well shown in Table I.

\* All measurements were made with a Lucas Comparator.



In ten animals the right vagus was then exposed in the neck and stimulated with a faradic current of sufficient strength to slow, but not to inhibit completely, the spontaneous rhythm of the auricle. Stimulation of the nerve was commenced a few cycles before the introduction of the induction shock and was continued for less than two seconds unless an abnormal rhythm developed. Typical observations are shown in Table II. Stimulation of the vagus brought about a conspicuous shortening of the refractory period. In no

TABLE II

*Refractory Period of the Dog's Auricle. Spontaneous Rhythm. Influence of Vagus and Atropine. (R-S Intervals in Seconds)*

Dog No.....	19		16		32		29	
Auricular rate.....	156	70*	167	48*	140	168	187	169
	Control	During vagal stimulation	Control	During vagal stimulation	Control	After atropine	Control	After atropine
Refractory period				0.0848				
			0.1418	0.0771		0.1400	0.1065	
	0.1306	0.0806	0.1312	0.0685	0.1044	0.1333	0.1048	0.1210
	0.1270	0.0737	0.1175	0.0608	0.1013	0.1298	0.0985	0.1150
	0.1245	0.0655	0.1063	0.0559	0.0964	0.1280	0.0918	0.1108
	0.1195	0.0595	0.0992	0.0530	0.0950	0.1257	0.0874	0.1067
	0.1182	0.0486	0.0966		0.0938	0.1225	0.0863	0.1041
	0.1170	0.0437	0.0946		0.0880	0.1214	0.0835	0.1012
	0.1124		0.0890		0.0820	0.1155	0.0790	0.0955
			0.0816				0.0760	

\* Average rate.

instance did the refractory period under vagus stimulation exceed in length 0.0629 sec. Indeed in six experiments it lay below the minimum range of the stimulating pendulum (0.0475 sec.). These results agree with those of Lewis, Drury and Bulger (1).

The refractory period was measured in seven hearts after the intravenous injection of atropine (0.001 gm. per kg. body weight). Under the action of this drug it was prolonged well beyond the value obtained in the control observations. The average increase in length amounted to about 20 per cent (See Table II).

TABLE III

*Refractory Period of the Dog's Auricle. Spontaneous Rhythm. Influence of Adrenalin after Atropine. (R-S Intervals in Seconds)*

Dog No.....	21			27		
Auricular rate.....	82*	167	215	135	145	190
	During vagal stimulation	Control	After atropine followed by adrenalin	After atropine	Control	After atropine followed by adrenalin
Refractory period	0.0765		0.0916		0.1312	
	0.0714		0.0887	0.1604	0.1278	0.0903
	0.0670	0.1165	0.0845	0.1587	0.1248	0.0854
	0.0609	0.1142	0.0806	0.1567	0.1215	0.0787
	0.0545	0.1095	0.0752	0.1503	0.1206	0.0739
		0.1063	0.0710	0.1454	0.1155	0.0701
		0.1030	0.0655	0.1425	0.1105	0.0656
		0.0984	0.0619	0.1412	0.1078	0.0624
				0.1365		

\* Average rate.

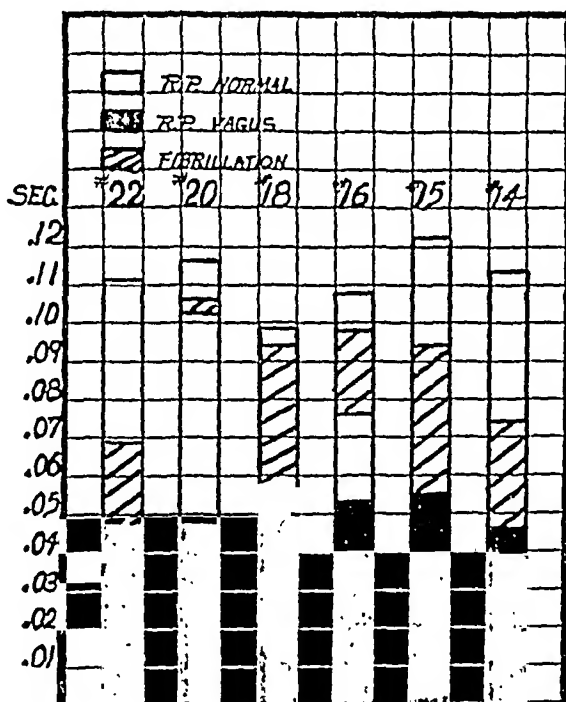
TABLE IV

*Refractory Period of the Dog's Auricle. Spontaneous Rhythm. Influence of Vagus, Atropine and Adrenalin. (R-S Intervals in Seconds)*

Dog No.....	20			
Auricular rate.....	120	54*	118	176
	Control	During vagal stimulation	After atropine	After adrenalin
Refractory period	0.1314			
	0.1276	0.0870		0.0906
	0.1238	0.0800		0.0887
	0.1222	0.0670	0.1482	0.0828
	0.1190	0.0540	0.1435	0.0819
	0.1165	0.0475	0.1396	0.0806
	0.1114		0.1340	0.0735
	0.1092		0.1307	0.0693
	0.1012		0.1245	

\* Average rate.

A slow intravenous infusion of adrenalin (1/1,000,000 in normal saline) after the vagus endings had been paralysed with atropine, caused acceleration of the spontaneous rhythm and shortening of the refractory period. Six such observations were made. The refractory period showed an average reduction of 40 per cent as compared with the control measurements but was never shortened to the extent



TEXT-FIG. 1. R-S intervals, at which auricular fibrillation followed a single stimulus, compared with the control refractory period and that under vagal stimulation.

produced by vagus stimulation (See Table III). In Table IV are shown the alterations in the refractory period in a single auricle produced successively by vagal stimulation, by atropine and by adrenalin.

It was repeatedly observed that a stimulus introduced during vagal stimulation, shortly after the end of the refractory period, was followed not by a single response, but by auricular fibrillation. This was not encountered in the control observations before stimulation of the vagus or following atropine or adrenalin.

Such results have been previously reported by DeBoer (6) and others in hearts deprived of their blood supply or poisoned with various drugs. Lewis, Drury and Bulger (1) have called attention to the occasional occurrence of such a disorder of rhythm during refractory period determinations while the vagus is being stimulated. The development of this irregularity in our series of experiments following a stimulus applied well out on the auricular appendix is of particular interest. The fact that there is no measurable gap between the stimulus and the beginning of the re-entrant rhythm (as shown in Fig. 2) indicates, we believe, that it rises at that point and not in a ring of muscle at the base of the auricle.

Moreover this rhythm consistently followed stimuli which, in the absence of vagus stimulation would have fallen within the refractory period, Text-fig. 1. Due, however, to the shortening of the refractory period consequent upon stimulation of the vagus, they fell upon tissue which was excitable but in which conductivity had, presumably, not yet returned to normal. Hence as long as vagal stimulation was continued there existed in the auricular musculature conditions favorable to a re-entrant rhythm. The fact that, in the normal heart under conditions of increased vagal tone, an extrasystole occurring early in diastole may set up a re-entrant rhythm suggests to us a possible explanation, in some instances at least, for the genesis of auricular fibrillation.

#### SUMMARY

1. A method is described for determining the refractory period of the dog's auricle during the normal sinus rhythm. The advantages of the method are:

(a) The total stimulating effects of repeated induction shocks are avoided.

(b) The action current is recorded from a point one millimeter or less from the point of stimulation.

(c) Alterations in the spontaneous rate of the auricle do not interfere with the accurate determination of the refractory period.

2. The values obtained for the normal refractory period and the changes produced by atropine and by stimulation of the vagus agree closely with those of previous observers.

3. The automatic features of the method make possible the determination of the refractory period under adrenalin. This drug brings about a distinct shortening of the refractory period but less than that produced by stimulation of the vagus.

4. During vagal stimulation a single induction shock, introduced soon after the end of the refractory period, frequently produces auricular fibrillation. The cause of this irregularity is discussed and its relation to clinical auricular fibrillation is suggested.

## APPENDIX

### *Amplifier and Timing Device*

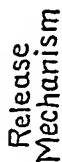
By HAROLD A. WHEELER, PH.D.

The complete apparatus comprises a five-stage resistance-coupled vacuum-tube amplifier, greatly amplifying the cardiac action-currents, together with a magnetically-actuated mechanical system automatically introducing a stimulating induction shock. The arrangement of the apparatus is illustrated schematically in Text-fig. 2. The resistance values are given in ohms, the capacitance values in microfarads. A six-volt storage battery and two 90-volt dry batteries supply the necessary power. The recording electrodes are connected to the electrocardiograph through an intermediate 10,000-ohm resistor. Independently of the amplifier, the action-current deflection on the galvanometer is adjusted to a value between one-half and two millivolts, the apparatus being designed to operate on this terminal voltage of the galvanometer without further adjustment. The galvanometer string is at no time exposed to the output of the amplifier.

The apparatus performs two functions at the same time; it amplifies the action current to a value of more than 20 volts, and stores, and slowly discharges, energy so that the amplified voltage has a duration of approximately 1/10 second as compared with the 1/50 second duration of the unamplified action current. The amplification of the potential difference applied is thus about 200,000 times, and represents about 10,000,000 times the energy of the action current.

The amplified current flows through a 10-milliamperere meter and a 10,000-ohm magnet. During adjustment for operation, a switch across the magnet is closed and the magnet is not actuated. The amplifier responds only to a positive potential applied to the first amplifier tube; the reversing switch in the recording electrode circuit must, therefore, be adjusted by trial to give the sharpest deflection of the meter pointer.

The mechanical system is activated upon opening the magnet switch. Each amplified action-current impulse thereafter instantly moves the magnet armature. This armature carries an escapement such that the second impulse releases a hook, which, in turn, sets free a falling arm. The falling arm operates a trip-switch,



H.A.W.  
4-22-29

## HEART AMPLIFIER AND ACCESSORIES FOR DETERMINATION OF REFRACTORY PERIOD.

TEXT-FIG. 2

opening the primary circuit of an induction coil. The stimulating electrodes thereupon receive a shock from the secondary circuit of the induction coil. The interval between the second action-current impulse and the induction shock can be adjusted to any value between  $1/20$  and 1 second. The speed of the falling arm is determined by the position of two balancing weights. The angular position of the trip-switch is also adjustable so that the switch can be operated at any time during the period of the falling arm. For the present experiments, a calibration curve was prepared showing the interval at all angular positions of the trip-switch.

The instrument is designed to actuate the magnet armature in response to an action-current impulse of  $1/2$  millivolt for  $1/50$  second. The grid condensers and grid resistors thus give the entire amplifier a discharging time constant of about  $1/10$  second, which is sufficiently short to restore the amplifier to normal very quickly after a short impulse of several millivolts.

The condensers in parallel with the first two plate circuits are proportioned to give the amplifier a charging-time-constant of about  $1/50$  second. This is sufficiently short to respond quickly but sufficiently long to prevent disturbances in the amplifier at audio or radio frequencies. These parallel condensers also serve to increase the duration of the amplified impulse to about  $1/10$  second—giving the magnet ample time to respond and preventing a quick rebound of its armature. A 90-volt battery common to all five plate circuits would give feedback-coupling from the fifth to the first plate circuits with resulting low frequency oscillations; therefore two separate 90 volt batteries are necessary. With a normal grid bias on the last tube, the magnet may be actuated by random fluctuations in the plate current of the first tube ("flicker effect") amplified to a high degree. This grid bias is, therefore, operated at a high negative value, giving a normal plate current of about  $1/2$  milliamperes. The magnet armature is adjusted to respond only to 2 milliamperes or more, so that it is not actuated by fluctuations. Some difficulty may be experienced with radio-frequency disturbances picked up by the wires connected to the first grid. This is prevented by the condenser shunting the first grid circuit, and by enclosing the entire amplifier with its batteries in a galvanized-iron box. This condenser, and the filament circuit of the first tube, are connected to the box by a short wire. Mechanical agitation has no effect upon the amplifier.

The release mechanism comprises the magnet with its armature, escapement, and trip-switch, shown schematically in Text-fig. 2. The magnet has a laminated "E" core. The middle leg of the core has a cross section of one square inch and carries a 10,000 ohm coil of 40 gauge copper wire. The armature is made of  $1/32$  inch iron sufficiently large to cover the three poles and is supported at its base on a horizontal pin parallel to the line of poles. It carries an escapement which, on the second impulse, releases a light hook. This release on the second impulse is important because it avoids the confusion which might occur if the magnet switch were opened during the first impulse.

The falling arm is of light construction, one foot long, and pivoted at the center. It carries two weights, whose positions are adjustable by a threaded rod along the

length of the arm. A shock-absorber catches the arm after a 90 degree fall from its initial position. The arm, when released from the hook by the magnet, engages the trip-switch. The angular position of this switch is adjustable and is indicated on a circular scale.

The trip-switch details are shown schematically on an enlarged scale. It is made up of two insulated cylinders carrying metal contact segments. The primary contactor is engaged directly by the falling arm. The secondary contactor floats on the same pin, but is engaged by a pin on the former, and moves only during the latter part of the motion of the primary contactor. When the arm is raised the primary circuit of the induction coil is first closed and the secondary shock absorbed, then the secondary circuit is opened ready for the experiment. When the arm falls, the primary circuit is first opened, producing an induction shock at the stimulating electrodes, and the secondary coil is shunted.

Thus all mechanical operations are performed by merely raising the falling arm. The trip switch is set and the induction-coil-battery circuit closed. The hook is pushed back and automatically sets the escapement on the armature. Observations can thus be made in rapid succession.

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#### EXPLANATION OF PLATE 8

FIG. 1. Curves recorded from the point of stimulation; (a) during control observation, and (b) during vagal stimulation. The stimulus appears as a thickening on the rising limb of the wave.

FIG. 2. Fibrillation of the auricles following a single induction shock during stimulation of the vagus. Stimulation begun at A and ended at B.





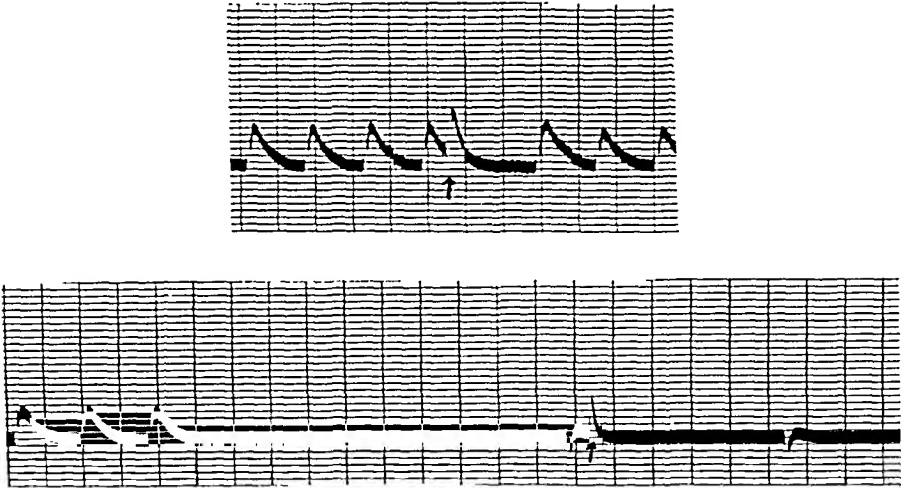


FIG. 1

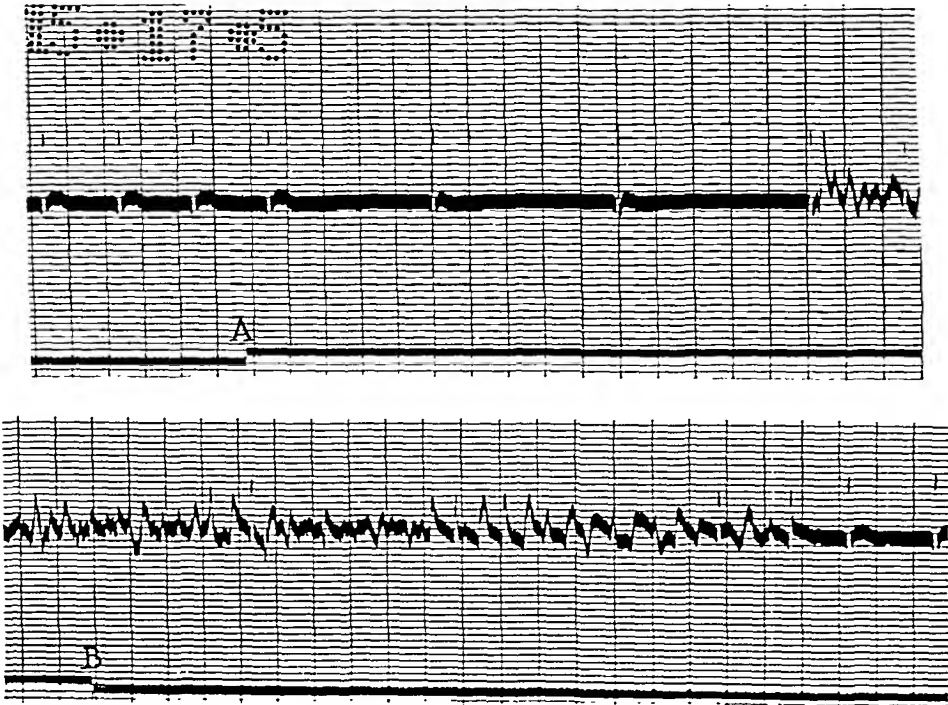


FIG. 2



## STUDIES ON VITAL STAINING

### I. SOME PROBLEMS IN COLORIMETRY. THE QUANTITATIVE ANALYSIS OF MIXTURES OF COLORED SUBSTANCES IN SOLUTION

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#### INTRODUCTION

In the course of some experiments on vital staining a need arose for a method for quantitative analysis of various dyestuffs when in solution along with other colored substances which would interfere with measurement in any of the ordinary standard colorimeters. This problem was finally solved by use of the spectrophotometer. With this instrument one may measure the amount of light absorbed by the solution in any portion of the spectrum. A blue dye transmits blue light but absorbs strongly in the red, and by measuring the amount of this absorption in the red end of the spectrum one may calculate how much of the dye is present in the solution. Conversely, a red dye transmits red light but interferes with the passage of blue and green light, so that the dye concentration may be determined by ascertaining to what extent blue light is absorbed. In case both dyes are present simultaneously each may be determined separately by taking measurements in the two portions of the spectrum where each has a more or less specific effect. It is true that the absorption is not entirely limited to one particular region of the spectrum in the case of either dye, but in suitable cases the correction is small and can be applied in the way outlined below. Other workers (1) have considered this problem of color analysis, but from a somewhat different point of view. It is the purpose of the present paper to describe the method for the analysis of colored mixtures in general terms and to give simple equations which are applicable to the problem studied in the papers to follow. These same equations and the same method in general should be

applicable to a wide variety of problems. Spectrophotometric analysis of the sort here discussed has not received the attention which it deserves from biologists. The quantitative analysis of such mixed colored solutions should find many applications in biology. Dye solutions may be studied quantitatively, even though hemoglobin, bile pigments or urinary pigments may be present as contaminations—a condition often present in biological studies. Dyes excreted into the urine can be measured despite the presence of urinary pigments or even blood. Quantitative analyses by means of color reactions are often unsuccessful on account of extraneous pigments or side reactions. In such cases spectrophotometric analysis might be used to rule out the interfering effect of the foreign colored substance.

The method just outlined will be illustrated by the use of two dyes, brilliant vital red and Niagara sky blue, for they are the ones used in the work to be reported later, but the method of analysis of colored mixtures is applicable to a wide variety of substances, the chief condition being that the two substances mixed must be of different colors. Certain limitations of the method will be discussed more in detail later.

### *The Analysis of Simple Colored Solutions*

There are several types of spectrophotometers which differ in various details. In all types a beam of white light is broken up by the instrument into a continuous spectrum of violet, blue, green, orange and red. If a glass cell of known thickness containing the unknown colored solution is placed in the beam of light, certain portions of the spectrum will be darkened more than others. By means of a slit in the eyepiece the view may be limited to any particular portion of the spectrum and the amount of dimming can be measured by means which vary with different types of spectrophotometer.

For ideal solutions the Lambert-Beer law states that

$$(1) \quad i_1 = i_0 \cdot 10^{-ECh}$$

or,

$$(2) \quad \frac{i_1}{i_0} = 10^{-ECh} = T$$

where  $i_1$  is the intensity of light of any given wave length after the beam has traversed the colored solution,  $i_0$  is the intensity before traversing the solution.  $C$  is the concentration of the latter (here expressed in terms of mg. per liter),  $h$  is the thickness of the layer of dye solution (expressed in cm.) and  $E$  is a constant which

varies with the nature of the colored substance and with the portion of the spectrum examined. The ratio of  $i_1$  and  $i_0$  represents the fraction of the light transmitted and is independent of variations in intensity of the light source. In some types of instrument this fractional transmission,  $T$ , can be read off directly on the scale of the instrument or can be computed by relatively simple calculation.

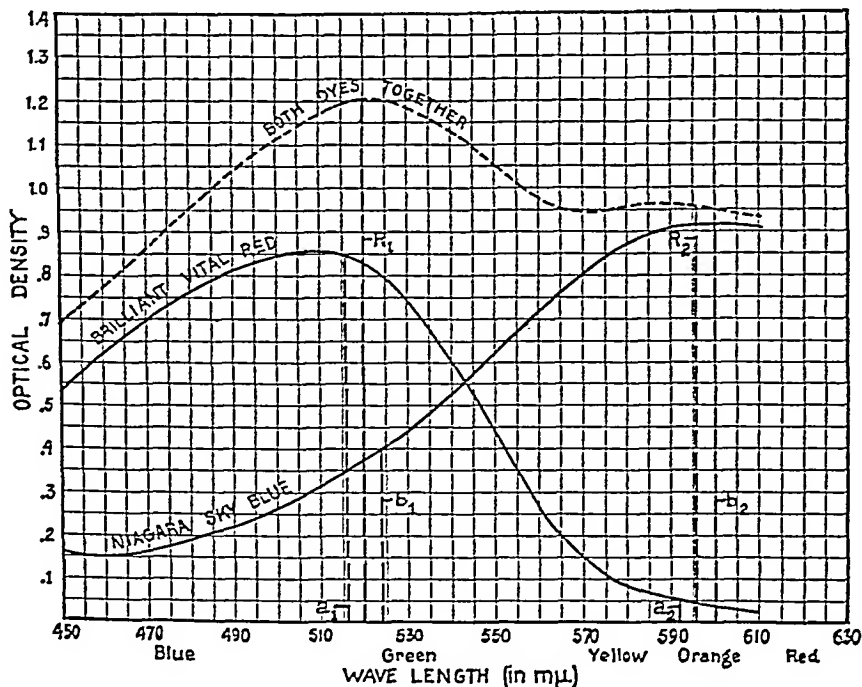


CHART 1A. Absorption curves calculated for 0.002 per cent solutions. Layer = 1 cm. thick.

Obviously our interest centers in the values of  $E$ ,  $C$  and  $h$ . These factors may be grouped together by letting

$$(3) \quad D = ECh$$

Then,

$$(4) \quad \frac{i_1}{i_0} = T = 10^{-D}$$

$D$ , the "optical density" of the solution may be looked upon as the obstructive power of the dye solution and is proportional to the concentration and to the

thickness of the layer. Its value varies in different parts of the spectrum depending upon the particular kind of light absorbed by that particular substance. In some types of instrument the calibration is such that  $D$  can be read directly on the scale of the photometer. In other instruments  $D$  must be calculated from the observed value of  $T$ . This calculation is very simple, since from equation 4 it is obvious that  $D = -\log T$ . In the Bausch and Lomb instrument used by us a control beam of light enters the instrument alongside the first beam and the two are matched by means of Nichol prisms. One glass cell containing the colored solution is placed in one of these beams of light. The other beam traverses a control cup filled with water. One should adopt the practice of reversing the cups and taking a second reading, thus avoiding errors arising from polarization and from slight maladjustment of the instrument. The readings of the Nichol prisms are shown in degrees. For purposes of precision the colored solution should be so diluted that the first reading lies between  $10^\circ$  and  $25^\circ$ . On reversing the cups a large angular reading is obtained (between  $65^\circ$  and  $80^\circ$ ). From these two readings one may calculate the optical density from the formula,

$$D = \log \cot \text{small angle} + \log \tan \text{large angle}.$$

Having determined the value of  $D$  one may calculate the concentration of the colored substance by means of formula 3. This latter step necessitates knowledge of the value of  $E$  for that particular substance in that portion of the spectrum, but this value is readily ascertained by making a preliminary analysis of the colored substance in pure solution. In Chart 1A are shown the absorption curves of the two dyes, brilliant vital red and Niagara sky blue. For reasons to be discussed later the dyes were made up in a mixture of saline and normal dog plasma. The different portions of the spectrum are plotted according to their wave lengths expressed in  $m\mu$  (1  $m\mu$  equals 10 Ångstrom units). The power of each dye to absorb light ( $D$ , the "optical density") is shown by the ordinates. It is seen that brilliant vital red absorbs almost none of the red light, but is quite effective in absorbing blue and green light. The maximum absorption is in the vicinity of 500–520  $m\mu$ . In quantitative analysis of this dye it is obvious that the greatest precision is obtainable by basing the calculation on readings taken in the region of the maximum density. We note that at 520 the "optical density" of a layer 1 cm. thick of the 0.002 per cent solution is 0.84. If we express concentration in terms of milligrams of dye per liter of fluid we find from equation 3 that the value of  $E$  is 0.042. In case of the blue dye the maximum absorption is in the region of 600  $m\mu$  and the value of  $E$  at this particular point is 0.046. Once these values of  $E$  have been determined one may proceed to the analysis of unknown solutions of these substances, using equation 3 for purposes of computation. Using this method of color analysis one obviates the necessity of having to prepare frequent standard solutions of these substances as in ordinary colorimetric work. One avoids unnecessary labor and at the same time is assured of results which are independent of uncontrollable variations in the standard.

*The Analysis of Two Admixed Colored Substances*

When one desires separate quantitative analysis of two colored substances present simultaneously in solution one may use the method previously mentioned, i.e., one may measure the absorption of light in the two portions of the spectrum where each dye has its characteristic absorption band. The situation is illustrated graphically in Chart 1A by the absorption curves of Niagara sky blue and brilliant vital red. In case both dyes are present simultaneously in equal amounts the mixture becomes purple and the absorption curve is the sum of the two individual curves and is shown by the dotted line. It is with such mixtures of colors that one has to deal in experimental work and it is our problem to deduce from this summation curve just how much of each of the two dyes is present in the mixture. For purposes of making this analysis we may choose to make readings at wave length 520 and 600, for in these regions one dye shows approximately its maximum absorption while the other shows nearly the minimum. Readings taken nearer the ends of the spectrum would be somewhat more ideal theoretically, but it is difficult in these regions to match the colors accurately in the spectrophotometer.

Referring to Chart 1 A it is seen that  $R_1$  and  $R_2$  can be measured in the spectrophotometer. We wish to ascertain the values of  $a_1$  and  $b_2$  so that we may calculate from them the concentration of each dye in the mixture.

Inspection shows that

$$(6) \quad R_1 = a_1 + b_1$$

$$(7) \quad R_2 = a_2 + b_2$$

But the form of the absorption curve of each of the two dyes is very specific. Hence

$$\frac{a_1}{a_2} = K_a, \text{ or } a_2 = \frac{a_1}{K_a}$$

$$\frac{b_1}{b_2} = K_b, \text{ or } b_1 = K_b b_2$$

where  $K_a$  and  $K_b$  are constants which can be determined for each dye by study of the latter in pure solution uncontaminated by the other dye. Substituting these values in the equations above

$$(8) \quad R_1 = a_1 + K_b b_2$$



$$(9) \quad R_2 = \frac{a_1}{K_a} + b_2$$

from which it follows that

$$(10) \quad a_1 = \frac{K_a R_1 - K_a K_b R_2}{K_a - K_b}$$

$$(11) \quad b_2 = R_2 - \frac{a_1}{K_a}$$

These values of  $a_1$  and  $b_2$  are "optical densities" and by substituting their values for  $D$  in equation 3 the concentration of each dye in the mixture can be calculated.

Before undertaking the analysis of colored mixtures one should plot accurate absorption curves of pure solutions of each substance. This gives the various constants needed and at the same time allows one to proceed wisely in choosing the points in the spectrum where measurements should be made in order to give the greatest possible precision. The relative quantities of the two substances present influence the accuracy of analysis in a way which will be evident at once. In general the substance can be determined more accurately when it occurs in large amounts in the mixture. However it is possible to measure small amounts of a substance in the presence of large amounts of another colored substance provided the absorption bands of the two substances are fairly well separated. Inspection of Chart 1A shows that brilliant vital red absorbs very little light at 600. The large absorption by Niagara sky blue at this point permits very accurate analysis of the latter. On the other hand at 520 the light absorption is by no means all due to brilliant vital red; hence the amount of the latter cannot be measured so precisely as would be possible if the Niagara sky blue absorption band were more strictly localized to the red end of the spectrum.

In all spectrophotometric analysis it has been found desirable to take readings at several points in the region of the spectrum chosen for special study. On plotting these points one will discover and correct small errors due to inexact matching of the colors. It is also wise to take readings in neutral portions of the spectrum so chosen that suspected contaminating substances will be detected when the curve is plotted. In our own experiments to be reported later we

were concerned with the analysis of dyestuffs in plasma, and under these circumstances readings were taken in parts of the spectrum where hemoglobin and bilirubin would be detected if present.

It would seem hardly necessary to remind workers in this field that hydrogen ion concentration of the solvent may totally alter the form of the absorption curve of the dissolved coloring matter. In some

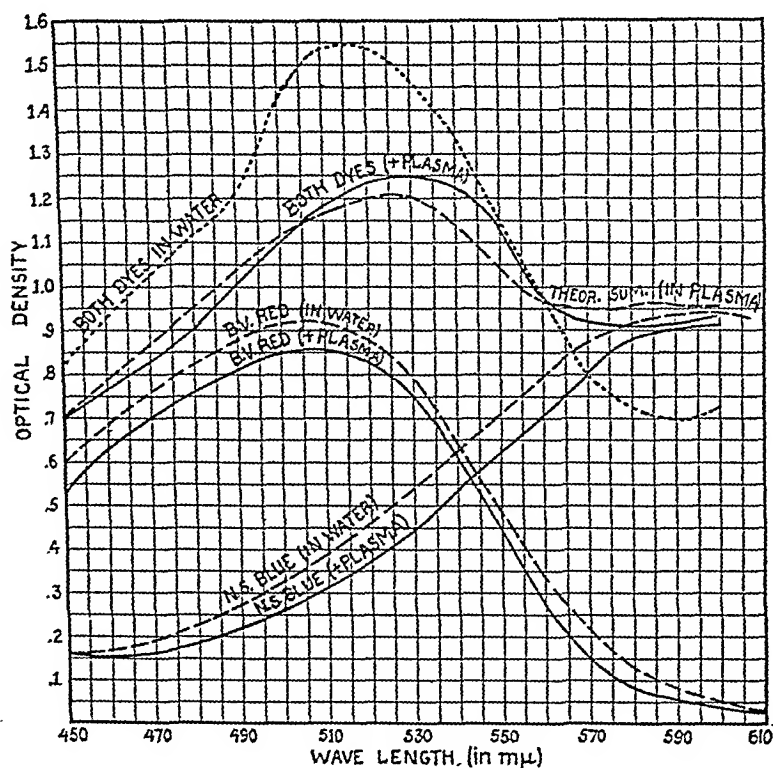


CHART 1B. Light absorption curves of brilliant vital red and Niagara sky blue (0.002 per cent solution) in water, and in plasma diluted with four parts of saline.

cases the salt concentration or protein concentration is also of great importance in its effect upon a dye. The preliminary control experiments should include a study of all such factors and their possible influence upon the color behavior of the dye. Okuneff (2), Seyderhelm and Lampe (3) and others have shown that whereas trypan blue dissolved in water is violet in color, the addition of plasma proteins

or egg-white gives the dye a more bluish color. In studying mixtures of colored substances the possibility of interaction between the two must also be kept in mind. In extreme cases precipitation may occur, but in other cases the reaction is much less evident and the only effect noted may be that the observed absorption curve of the mixture does not quite represent the summation of the absorption curves of the two components. A difficulty of this very sort arose in the case of mixtures of brilliant vital red and Niagara sky blue, and for a time it seemed as though it would be impossible to make quantitative studies of such mixtures. Curiously enough these difficulties existed only when the two dyes were brought together in aqueous solution. When a small amount of plasma protein was added to the mixture it was found that the combined absorption curve of the two dyes approached the theoretical summation curve in quite a gratifying manner. These facts are shown graphically in Chart 1B. The discrepancy from theory in pure aqueous solution is less marked in the blue than in other portions of the spectrum. In the green it amounts to about 30 per cent, and well over in the red near 590 the discrepancy is almost as great, though in the opposite direction. From the standpoint of quantitative analysis it is most fortunate that a little plasma protein will correct these abnormalities in light absorption and give values so closely approximating the theoretical summation curve shown by the interrupted line in the chart. It is rather remarkable that relatively small amounts of plasma need be added to bring about this result. Experiments have shown that one part of plasma to ten of the color mixture is adequate. The plasma of vitally stained dogs may contain large quantities of the two dyes and in such cases it may be necessary to dilute it preparatory to making spectrophotometric readings. In order to preserve an adequate concentration of the stabilizing proteins it is well to use saline mixed with normal dog plasma as a diluent. Normal plasma has a weak absorption curve of its own, but this may be ascertained in advance and proper correction made if necessary.

As yet we do not fully understand how the proteins bring about this stabilizing effect on the color absorption curve, though it is suggested that the proteins enter into some form of combination with the dyes and thus prevent or break up loose combinations which tend to form between the dyes themselves. It is noteworthy that either dye alone

in solution gives a color absorption curve which is largely independent of the presence or absence of plasma proteins. It would appear to be a combination of the dyes which is affected by the proteins.

It is clear that the effect on the dyes cannot be attributed to the buffer action of the plasma, for these particular dyes are relatively insensitive to changes in acidity unless very large amounts of acid are added. Furthermore, we can show that the two dyes made up together in selected inorganic buffer solutions show color absorption curves almost identical with those obtained in pure aqueous solution, and like the latter the deviation from the theoretical is quite marked unless plasma protein be added also.

#### SUMMARY

1. A spectrophotometric method is discussed which permits quantitative analysis of colored substances present in mixtures
2. Special attention is given to the analysis of mixtures of two dyes which are being used in a series of studies on vital staining.
3. It is shown that the method can be applied to quantitative analysis of mixtures of naturally occurring animal pigments or to mixtures of these with various other colored substances.
4. Certain limitations of the method and certain necessary precautions are discussed.

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## STUDIES ON VITAL STAINING

### II. THE REMOVAL OF BRILLIANT VITAL RED FROM THE BLOOD STREAM. DISTRIBUTION OF DYE BETWEEN BLOOD STREAM AND BODY TISSUES

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(Received for publication, November 16, 1929)

#### INTRODUCTION

In recent years there has developed a voluminous literature which deals with the phagocytosis of foreign materials by various body cells. Ink, bacteria and various dyes have been introduced into the living animal until the tissues were heavily laden with these substances. There has been much discussion about the possibility that one may saturate the tissues to the point that certain of their physiological powers are destroyed, or at least impaired. References to the literature may be found in the monographs of Aschoff (1) and of Börner-Patzelt, Gödel and Standenath (2).

These foreign substances with which we are concerned are taken up and stored in granular form within a vast number of cells, some of which are endothelial in nature, while others are wandering or fixed cells of connective tissues. Details concerning the histological aspects of the problem will be given in a subsequent paper (3). The phagocytic cells scattered throughout the body have been referred to collectively as the "reticulo-endothelial system." Many and varied physiological activities have been ascribed to them. Much has been written of their activity in freeing the blood stream and the tissues generally of objectionable particulate matter, such as bacteria, dead cells and cell debris. There is much evidence to indicate an ability on their part, to transform hemoglobin to bile pigment. It has been supposed that they may play a part in fat metabolism and by some they are thought to be concerned in the elaboration of immune bodies. Still other supposed activities are discussed in the monographs cited above. Many of these theories rest on the most insecure basis. Changes in pigment metabolism, in immune body balance, etc. are often observed to follow injection of certain foreign substances such as ink or carmine. Those who think that ink or carmine paralyze or "block" the reticulo-endothelial system will attribute all of these reactions to changes in the latter, and, totally disregarding possible changes in other organs or tissues, they will find no end of "functions" of this system. Other

workers, more interested in settling the question of "blockade" than in establishing new functions of the reticulo-endothelial system are sometimes rather hasty in assuming that the system has this or that function, and from changes observed they may draw erroneous conclusions in regard to the existence of a "blockade."

Thus, each of these groups of workers assumes the point which the other tries to prove by experiment. It is easily seen that such arguments are in a circle and are utterly futile. We would suggest that much desirable information concerning "blockade" may be obtained by careful study of reactions in which the rôle of the reticulo-endothelial system cannot be questioned. In the present state of knowledge we feel that much uncertainty prevails concerning the great majority of the "functions" of these cells. We feel that more is to be gained by a study of their long-known function of phagocytosis, or "storage" of dyes or other materials.

In the case of many dyes this process can be followed with the microscope, but unfortunately the purely morphological studies are almost entirely qualitative. They furnish little information about the degree or the speed of phagocytosis. The rate at which dyes leave the blood stream gives a better quantitative measure of such activity. However, many dyes and other substances in current use disappear quite rapidly from the blood stream and in many cases the particles tend to agglutinate in capillaries even before they are taken up by phagocytes. Other substances pass out rapidly through the bile or urine or both. Obviously in none of these cases is the rate of disappearance from the blood stream a trustworthy criterion of phagocytic activity. It should be stressed that most of the substances used in the past have had these defects to a striking degree. As yet no ideal test dye has been discovered, though a few approach the ideal much more closely than the rest.

A large number of dyes has been studied by Dawson, Evans and Whipple (4) and much valuable information has been obtained concerning the rate at which they leave the circulating blood. One of these, brilliant vital red, has already received further study from this standpoint by the present author (5). The dye is relatively non-toxic. It is not readily precipitated in neutral or nearly neutral solutions by the salts common in the animal body. The elimination through the kidneys is negligible. However, the dye is largely eliminated through the bile over the course of several days, but in the meantime the tissues become deeply stained and microscopic examination shows the phagocytes everywhere to contain beautiful accumulations of the red dye. During the first 2 or 3 days following injection the amount lost through the liver accounts for distinctly less than half of the dye which leaves the blood stream. The rest is taken up by the tissues, largely within phagocytic cells.

In an earlier paper of this series (5) a study was made of the rate at which the dye leaves the blood stream, and curves were plotted to show the relations graphically. The concentration in the blood stream falls off rather rapidly during the first few hours, but much more slowly later on (see also Chart 2C of the present paper). At the end of about 48 hours considerable dye still remains in circulation and from this point on the dye is so slowly eliminated that the curve is almost horizontal. No doubt the curve would approach the horizontal still more closely were it not that a small amount of dye is continuously being lost by excretion into the bile. The tissues are now deep red and it is clear that they are not taking up dye as readily as they would do in their normal unstained condition.

It might have been anticipated that the phagocytes would ingest dye quite rapidly at first when the plasma and lymph about them contain such large amounts of dye, and this is quite in accord with general experience, but there seems to be little recognition of the fact that the phagocytic activity slows up long before all of the dye has been ingested. With larger doses, phagocytosis also slows up but leaving still larger quantities of dye in circulation than before, and along with this one notes that the phagocytes contain more dye. To explain these facts it was suggested that an equilibrium is established between dye in cells and dye in the fluids, and that the diminution in phagocytic activity is in part merely apparent, for renewed activity is observed if we disturb the equilibrium by injecting more dye. Very clearly this concept has important bearings on the question of "blockade" of the body phagocytes and it is of great importance to know whether these dye-laden cells will remove newly injected dye as rapidly as though they themselves were free of dye. Our initial observations about to be presented seemed to indicate that some impairment really exists, for we noted that after a course of vital staining new offerings of dye leave the blood stream less rapidly than normal. We found also that large doses of dye do not leave the plasma as rapidly in proportion as do smaller ones. But certain observations on bile fistula dogs placed these experiments in a new light, and it now seems that the retention of dye in the plasma is associated with inefficient liver elimination which is seen only when large amounts of dye are given. We can offer no evidence to indicate that the liver



tissue is actually injured by the dye. It may be entirely within the realm of normal physiology that 25 per cent of a small dose of dye passes into the bile in 24 hours whereas only about 10 per cent of a large dose will be excreted in such a period. The greater retention of dye within the body may well explain the retention of dye within the plasma, without our having to assume defective phagocytic activity on the part of the tissues. We wish to stress these observations and this interpretation in order to show the danger of assuming a blockade of phagocytes merely because dye leaves the blood stream unduly slowly. This view will be given further emphasis in a subsequent paper (6) where it will be shown that a small injection of India ink will inhibit almost completely for some days the excretion of brilliant vital red by the liver. The great retention of dye in the body during this period seems to be quite sufficient to account for the unduly great dye concentration in the plasma, and there is reason to believe that part of the retained dye has found its way into the tissues and that there is increased coloration there as well as in the plasma. There is little need to assume that the ink had inhibited the activity of the phagocytic system. These observations are in accord with our present contention that large amounts of brilliant vital red within the tissues need not inhibit the entrance into the phagocytic cells of newly injected dye. The disproportion in liver excretion seems to account for all of the findings which might be taken to indicate such impairment in phagocytosis.

### *Methods*

Healthy adult dogs maintained on a mixed diet were used in all experiments. To allow accurate colorimetric measurement of dye in the plasma, feeding hours were so arranged that the plasma would show no lipemia during the morning hours when samples of blood were commonly taken for analysis. The dogs had free access to water at all times. The dye used was brilliant vital red which was obtained from the National Aniline and Chemical Company. Dye from the same bottle was used throughout the course of the experiments. At intervals a filtered two per cent aqueous solution was made up. In each case the color intensity of this stock solution was checked spectrophotometrically just after preparation and also at intervals later on, so that constancy in preparation and absence of fading could be demonstrated.

Blood for analysis was collected in graduated 15 cc. centrifuge tubes containing 2 cc. of a 1.6 per cent sodium oxalate solution to prevent clotting. The tubes

were centrifuged and the amount of plasma read off on the tube, thus making it possible to correct for the dilution occasioned by the 2 cc. of oxalate solution previously added. The amount of dye in the plasma was determined by means of the spectrophotometer. Details of this method of analysis are given in a previous article (7). In all cases the concentration of dye in the plasma is expressed in terms of milligrams of dye per liter of plasma.

#### EXPERIMENTAL

If brilliant vital red be injected into the blood stream, the blood plasma taken several minutes later will be found to be bright red. If the amount of dye in such plasma be measured by colorimetric means we find that the concentration is almost exactly proportional to the amount of dye injected. With given dosage, proportional to body weight, this original dye concentration varies somewhat in different dogs depending on variations in blood volume. If such a standard dose be adopted as routine, it is found that this original high dye concentration is not maintained, but the concentration falls off, so that only 15-25 per cent of the dye remains 24 hours later. From this point on the fall is much more gradual, and we believe that this slowing up is related to the fact that the tissues are becoming stained with dye and on this account do not take up dye from the fluids as readily as normal. To be sure, the amount of dye now in circulation is not large, but we know that normal unstained tissues will attract such small amounts of dye quite readily, as one can demonstrate by injecting small doses into normal unstained dogs. This delay on the part of the stained tissues has been considered briefly in a previous publication (5). It was suggested that dye passes from plasma to tissues until an equilibrium partition is approached. We may believe that the amount of dye in the blood stream would finally become quite stationary were it not that a small amount of dye is constantly spilling over into the bile and is lost from the body.

*Dog 24-74.* Shepherd, 30 kgm.

May 23, 1927. Thirty cubic centimeters of a 2 per cent solution of brilliant vital red injected intravenously. Five minutes after the injection the concentration of dye in the plasma was 440 mg. per liter plasma; after 1 hr., 370; 6 hrs., 250; 24 hrs., 130; 48 hrs., 42; 72 hrs., 15.

May 26 to June 2 inclusive, daily injections of 30 cc. of a 2 per cent solution of the same dye. Five minutes after the last injection the plasma contained 670

mg. dye per liter plasma; 6 hrs. later, 525; 24 hrs., 335; 48 hrs., 190. Immediately following the collection of this last sample 30 cc. more of the dye were injected. Five minutes after this injection the plasma contained 540 mg. dye per liter plasma. After 6 hrs., 370; 24 hrs., 270; 48 hrs., 160; 72 hrs., 100.

Several months later almost all of the dye had been eliminated from the body and the tissues were again in a normal condition, suitable for further experiments.

August 3, 1927. Thirty cubic centimeters of a 2 per cent solution of the dye were injected intravenously. Five minutes later the plasma contained 415 mg. dye per liter plasma. One hour later, 335 mg.; 6 hrs., 245; 24 hrs., 84; 48 hrs., 30; 72 hrs., 19.

August 6 to 13 inclusive, daily injections of 30 cc. of the 2 per cent dye. Five minutes after this injection the plasma contained 740 mg. dye per liter plasma; 6 hrs. later, 550; 24 hrs., 360; 48 hrs., 185; 72 hrs., 102; 96 hrs., 67; 120 hrs., 45.

*Dog 24-96.* Shepherd, 18 kg.

May 24, 1927. Twenty cubic centimeters of a 2 per cent solution of brilliant vital red injected intravenously. Five minutes after the injection the concentration of dye in the plasma was 470 mg. dye per liter plasma; after 1 hour, 410; 6 hrs., 310; 24 hrs., 160; 48 hrs., 103; 72 hrs., 62.

May 27 to June 3 inclusive, daily injections of 20 cc. of the 2 per cent dye solution. Five minutes after the last injection the plasma contained 990 mg. dye per liter plasma; after 6 hrs., 735; 24 hrs., 520; 48 hrs., 420; 72 hrs., 300; 96 hrs., 205; 120 hrs., 180.

After several months had elapsed and the tissues had rid themselves of almost all of the dye the animal was used for the following experiments.

August 6, 1927. Twenty cubic centimeters of the 2 per cent dye solution were injected intravenously. Five minutes later the plasma contained 480 mg. dye per liter; after 1 hr., 430 mg. dye; 7 hrs., 295; 24 hrs., 138; 48 hrs., 76; 72 hrs., 48.

August 9 to 16 inclusive, daily injections of 20 cc. of 2 per cent dye solution. Five minutes after the last injection the plasma contained 830 mg. dye per liter plasma; after 6 hrs., 740 mg.; 24 hrs., 575; 48 hrs., 320. Immediately following the collection of this last sample 20 cc. more of the dye solution were injected. Five minutes after this injection the plasma contained 695 mg. dye per liter plasma; after 6 hrs., 625 mg.; 24 hrs., 390; 48 hrs., 250; 72 hrs., 210.

*Dog 25-16.* Airedale, 23 kgm.

May 22, 1927. Twenty cubic centimeters of a 2 per cent solution of brilliant vital red injected intravenously. Five minutes after the injection the concentration of dye in the plasma was 400 mg. per liter plasma; after 1 hr., 325 mg.; 6 hrs., 240; 24 hrs., 105; 48 hrs., 54; 72 hrs., 31.

May 25 to June 1 inclusive, daily injections of 20 cc. of a 2 per cent solution of the same dye. Five minutes after the last injection the plasma contained 790 mg. dye per liter plasma; 6 hrs. later 670; 24 hrs., 450; 48 hrs., 325. Immediately following the collection of this last sample 20 cc. more of the dye were injected. Five minutes after this injection the plasma contained 660 mg. dye per liter plasma. After 6 hrs., 530; 24 hrs., 375; 48 hrs., 280; 72 hrs., 200.

Several months were now allowed to elapse so that the dye might be eliminated from the body and the tissues become pale once more in preparation for the experiments to follow.

August 4, 1927. Twenty cubic centimeters of a 2 per cent solution of the dye were injected intravenously. Five minutes later the plasma contained 340 mg. dye per liter; 1 hr. later, 320; 6 hrs., 205; 24 hrs., 95; 48 hrs., 71; 72 hrs., 28.

August 7 to 14 inclusive, daily injections of 20 cc. of the 2 per cent dye. Five minutes after this injection the plasma contained 700 mg. dye per liter; 6 hrs. later, 510; 24 hrs., 355; 48 hrs., 225; 72 hrs., 155; 96 hrs., 123; 120 hrs., 100.

*Dog 25-29.* Collie, 28 kg.

May 21, 1927. Twenty-four cubic centimeters of a 2 per cent solution of brilliant vital red injected intravenously. Five minutes after the injection the con-

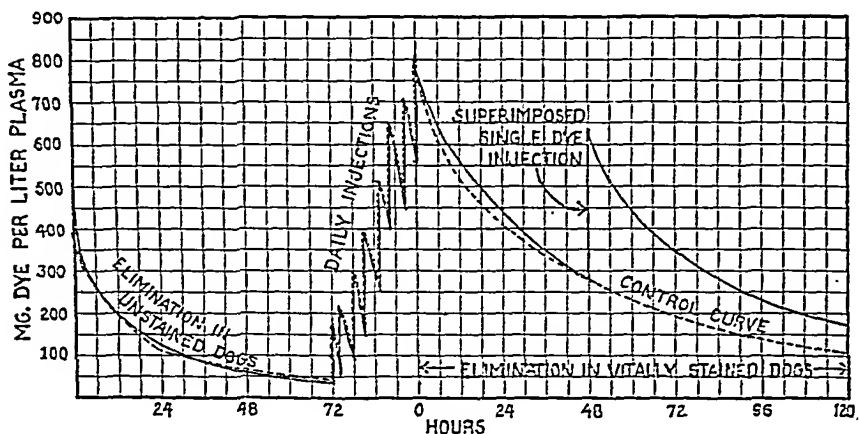


CHART 2A. Concentration of brilliant vital red in plasma (average of four experiments).

centration of dye in the plasma was 360 mg. per liter; after 1 hr., 340; 6 hrs., 280; 24 hrs., 114; 48 hrs., 59; 72 hrs., 43.

May 24 to 31 inclusive, daily injections of 24 cc. of a 2 per cent solution of the same dye. Five minutes after the last injection the plasma contained 806 mg. dye per liter plasma; 6 hrs. later, 625 mg.; 24 hrs., 405; 48 hrs., 315; 72 hrs., 230; 96 hrs., 170; 120 hrs., 110.

After several months had elapsed and the tissues had rid themselves of almost all of the dye the animal was used for the following set of observations.

August 5, 1927. Twenty-four cubic centimeters of a 2 per cent solution of the dye were injected intravenously. Five minutes later the plasma contained 390 mg. dye per liter; after 1 hr., 370 mg.; 6 hrs., 240; 24 hrs., 114; 48 hrs., 62; 72 hrs., 49.

August 8 to 15 inclusive, daily injections of 24 cc. of the 2 per cent dye solution. Five minutes after the last injection the plasma contained 810 mg. dye per liter plasma; after 6 hrs., 605 mg.; 24 hrs., 425; 48 hrs., 280. Immediately following the collection of this last sample 24 cc. more of the dye were injected. Five minutes after this injection the plasma contained 620 mg. dye per liter plasma; after 6 hrs., 540 mg.; 24 hrs., 350; 48 hrs., 220; 72 hrs., 180.

In order to learn more about the behavior of dye-stained tissues we have made daily dye injections into a number of dogs until their tissues were quite heavily stained. Under these circumstances the

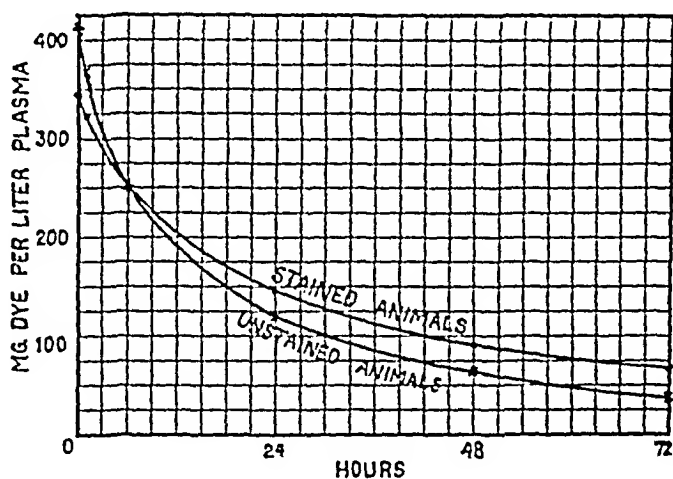


CHART 2B. Concentration of brilliant vital red in plasma (average of four experiments).

amount of dye circulating in the plasma may be very large. Even for 48 hours after the last injection rather large amounts of dye are still present in the plasma, but the rate at which the dye is leaving the circulating blood is very much reduced. The tissues are now deep red in color—a result of the dye granules present in the phagocytes. We might expect that these cells, partially filled with dye, would refuse further offerings, or at least would show a sluggish response. That they do not refuse altogether is most readily demonstrated by the injection of a test dose of dye. We are presenting 4 experiments which show this quite convincingly. The protocols are presented individually and Chart 2 A shows the average of all four dogs (Dogs 24-74, 24-96, 25-16, and 25-29).

The jagged peaks in the mid-portion of the chart illustrate schematically the rise in dye in the plasma accompanying the 8 daily intravenous injections of dye. At the end of this period the plasma was found to contain approximately 800 mg. dye per liter. The dotted control curve proceeding from this point shows that the dye leaves the plasma rather rapidly at first, but it is noted that 48 hours later the plasma still contains nearly 300 mg. dye per liter, and there are 100 mg. per liter at the end of 120 hours. It is seen that the curve is rapidly approaching a plateau. We believe that the elimination of dye by the liver is largely responsible for the fall noted in the last few hours of this curve. The tissues are still deep red. This is well seen in the skin and mucous membranes.

If several months are allowed to elapse the tissues will become almost entirely free of dye and the experiment may be repeated with almost identical results. Such a duplication is shown by the continuous line in this same chart. As in the control experiments it will be noted that following 8 daily injections the plasma again contained about 800 mg. of dye per liter, and that from this point on the two curves are very similar, and as before described the curve begins to flatten out decidedly after about 48 hours, and as before we also note that the tissues are deep red.

At this time a large test dose of dye was injected in order to ascertain how readily these dye-laden tissues would dispose of additional amounts of dye. As a result of this superimposed injection the dye concentration in the plasma promptly rose from about 300 mg. per liter to somewhat over 600. The curve from this point on shows rather rapid elimination at first, proving beyond doubt that the phagocytic activity was far from being abolished by the presence of the dye which these cells contained. We note, however, that the initial rapid loss of dye from the plasma is not maintained and the curve ultimately approaches a plateau which is nearly twice as high as that of the control curve shown by the dotted line.

We may look upon the space between these two curves as representing the reaction of the dye-stained tissues to this final superimposed dye injection. This difference may be measured at a number of points on the curves and the differences plotted separately. It is of great interest to compare the curve so obtained with a curve show-

ing the results in unstained dogs, and for purposes of comparison we have determined the normal elimination rate for these same dogs. The results are shown in the first portion of the chart, before the tissues had been stained by the daily injections. This normal curve is plotted separately in Chart 2 B and alongside this curve is the other which shows the arithmetical differences between the two curves just discussed. It will be observed that the slopes of the curves are decidedly different, and it is seen that when the dogs are vitally stained the dye leaves the plasma more slowly than normal, evidence

TABLE 21

	Dye per 1000 cc. plasma						
	5 min.	1 hr.	6 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Large dose (40 mg. per kg.)							
Dog 28-161.....	1020	885	700	410	225	147	71
Dog 28-158.....	850	710	570	325	152	94	42
Dog 28-146.....	865	745	530	305	165	84	52
Dog 28-107.....	1150	880	785	440	220	89	45
Av. all dogs.....	971	805	646	370	190	101	53
Small dose (8 mg. per kg.)							
Dog 28-161.....	157	150	118	56	26	8	
Dog 28-158.....	178	157	110	38	18	8	
Dog 28-146.....	154	133	95	37	21	7	
Dog 28-107.....	205	181	128	75	44	6	
Av. all dogs.....	174	155	113	47	27	7	

which might be accepted as proof that the tissues were becoming saturated with dye were it not that we know that liver excretion may greatly alter the amount of dye remaining in the body and hence in the plasma also. Even this factor of liver excretion could be disregarded if it were known that the amount of dye excreted into the bile were always directly proportional to the amount present in the plasma. Recent work in this laboratory shows quite conclusively that this is not the case. Part of this data will be presented in a later paper of this series (6) and part is left for future publication. We can show (6) that a small injection of India ink may have little or no effect on the volume of bile excreted, nor on the daily output

of bile salts and bile pigments, and despite this the liver may be almost completely unable to excrete brilliant vital red for 48 hours or longer. We know little of other conditions which may affect one function of the liver so specifically to the apparent exclusion of certain others. It is hoped that experiments now in progress will shed light on a field so little understood. Our recent studies also show that increasing

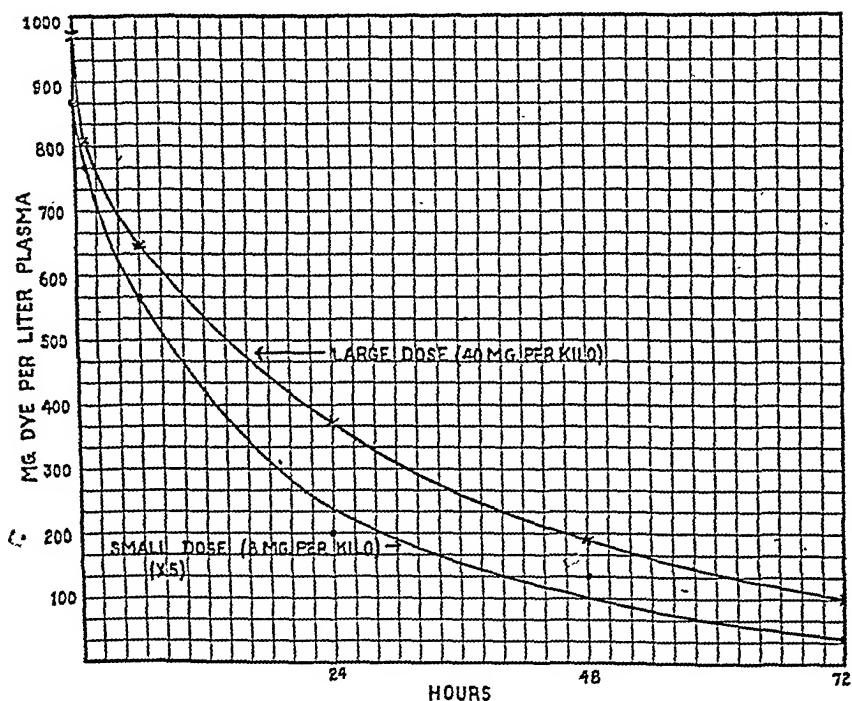


CHART 2C. Varying dose of brilliant vital red (average of four dogs—see Table 21).

the dye concentration in the plasma 5 fold does not increase liver excretion by a like amount. In fact the excretion is little more than doubled under these conditions. From this it must be clear that with large dye doses the dye is retained in the body relatively much more effectively. Such conditions must prevail in vitally stained animals where there is much dye in the plasma as well as in the tissues. The inefficient liver excretion under these conditions must go far to account



for the longer retention in the plasma of test doses injected to study the disappearance rate. In fact we believe that much of the retained dye does pass into the tissues, giving them as well as the plasma a much more intensive coloration.

Experiments like these to compare large and small injections have also been carried out on normal dogs without the bile fistula operation, and in these experiments we have made it a special point to determine the rate at which the dye leaves the blood stream. The results with 4 such dogs are given in Table 21. All gave essentially similar results, the averages of which are shown graphically in Chart 2C. With a large intravenous injection (40 mg. per kilo body weight) we observe that the plasma taken 5 minutes later is deep red in color. It contains nearly 1000 mg. dye per liter. Twenty-four hours later there are still nearly 400 mg. per liter and at the end of 72 hours about 100 mg. still remain in circulation. This curve is to be contrasted with one with a small dye injection made a number of weeks previously into these same dogs. At that time only 8 mg. per kilo had been injected. This dosage is only one-fifth that given in the later experiment, and in order to simplify comparison of the curves we have multiplied the values observed by 5, and in Chart 2C we have plotted the results alongside the curve resulting from the large injection. The curves thus brought to the same scale are quite similar for the first hour or so, but one observes that after that they tend to separate rather rapidly and at the end of the experiment one curve is almost twice as high as the other. It is noted that the marked lag is shown in the curve resulting from the large injection. It is quite obvious that dye does not leave the circulation five times as rapidly as with a dose one-fifth as large. We feel that the phagocytic activity may not be quite proportional to the dosage of dye and that with large dosage the undue delay in elimination may be due in part to this, but we are convinced that much if not all of the discrepancy between the two curves should be attributed to the fact that the liver does not excrete the large dose five times as rapidly as the smaller one.

#### DISCUSSION

The experiments just presented have shown that following multiple daily injections or, indeed, after a single large injection, much of the

dye remains in circulation for many days. The rapid elimination during the first hours stands quite in contrast to the slower elimination later on. After several days the amount in the plasma remains almost constant. A state has been reached such that the tissues take up little dye unless the concentration in the plasma is artificially raised by further dye injection. We feel that this relative inactivity of the tissues must be taken to mean that an approximate equilibrium has been established between dye in plasma and dye in the tissues. We know that this inactivity is not due to a lowering of the plasma dye concentration below a hypothetical functional threshold value, for we have shown that with small doses the normal unstained tissues will attack such amounts of dye with great vigor. The concept of an equilibrium between dye in plasma and dye in tissues is also borne out by work still in progress which goes to show that by rapid bleeding and transfusion of vitally stained dogs one can reduce the dye concentration in the circulating plasma to a very low level, and in such cases we have observed that dye passes back from tissues to blood stream, and for a number of hours the dye concentration in the blood stream increases. We can thus demonstrate the essential features of an equilibrium reaction, namely that the progress is reversible and the tendency is to resist or compensate for a displacement produced in either direction.

Quantitative concepts of dye partition between cells and the fluids which surround them received scant attention in the older literature. So much of the work concerns the permeability of cell membranes and here the problem of equilibrium is neglected. Other workers have been more concerned with the power of cytoplasm to dissolve dyes or to unite with them in one manner or another. Certain of these workers have had the equilibrium concept clearly in mind; others have been less specific, though in certain cases the concept seems to be implied. Attempts to compile quantitative data are almost completely lacking until very recently. Within the past few years Irwin (8) has made notable contributions to the study of the distribution of certain basic dyes between plant cells and the fluids in which they are immersed. Many factors were found which influence this distribution, but she is convinced that with constant conditions the amount of dye within the cell is proportional to the concentration of dye in the sur-

rounding fluid. Collander (9) has also made studies on plants, but with acid dyes. He gives much thought to the question of permeability, and the equilibrium concept is much less fully treated than in the work of Irwin. These quantitative studies of Collander and of Irwin involve plant cells only. The diffuse coloration of the sap of plant cells may well be comparable to the diffuse staining sometimes seen in animal cells, but it is difficult to relate this process to the granular storage in macrophages of certain acid dyes such as brilliant vital red. The brilliant and deeply colored granules which form within the macrophages are almost certainly not formed from simple staining of preexisting protoplasmic granules within the cell, though we must admit that the dye may exist in union with substances not previously segregated into discrete foci. Schulemann (10) and Evans and Scott (11) have stressed the view that the behavior of this group of dyestuffs in the body may be closely akin to the process of phagocytosis of small bits of particulate matter so familiar to all. There is the added feature that during the process of granule formation the cells must take the dye from solution and build it up into small concentrated microscopic aggregates. We wish to stress our observation that even in this type of staining, so closely related to phagocytosis, we can demonstrate the existence of an equilibrium between the dye in cells and the dye in the surrounding fluids. It is perhaps less surprising that such a principle holds where the dye seems more clearly to enter into union with preexisting materials within the cells. We should be more surprised to find it in the case of acid dyes where the granules are built up *de novo* within the cells by a process of concentration and storage far more elaborate in character.

We feel that studies concerning equilibria should be extended to other types of cells and to other dyes. Such studies should illuminate the questions regarding whether in various cases the dye within the cell is merely dissolved in fluids and lipoids of the cell or whether it exists in the form of chemical or physico-chemical combination with elements present in the cell. Very possibly no single rule holds for all dyestuffs and for all cells.

#### SUMMARY

Brilliant vital red injected into the blood stream of dogs is slowly taken up by phagocytes in various parts of the body, but eventually

an equilibrium is established, after which the concentration as measured in the plasma remains almost constant for long intervals of time.

This equilibrium can be disturbed by injecting more dye, and in this case the phagocytes resume ingestive activity, apparently with normal or nearly normal vigor. This activity continued until a rather large part of the newly injected dye has been removed, and as the reaction again slows up we note that both plasma and tissues contain more dye than before. It is difficult to be certain that the distribution ratio of dye between plasma and tissues remains unaltered with dosage, but evidence indicates that for non toxic doses, at least, this is approximately true.

This study of this partition ratio is complicated by the fact that the liver slowly excretes dye into the bile, and this helps to reduce the amount of dye in the body. Partial correction for this factor can be made by ascertaining the dye output in bile fistula dogs. These latter studies show that dye elimination into bile is *relatively less efficient* when large doses of dye are given to the animal than with smaller dosage. This undue retention of dye in the body with large dosage helps to maintain the dye concentration in the plasma at unduly high levels. These peculiarities in liver excretion have an important bearing on liver physiology in general, and in addition they also have an important application in connection with the theory of "blockade of the reticulo-endothelial system." It is now obvious that prolonged retention of dye in the blood stream does not of itself prove that this group of phagocytic cells is "blocked" against the entrance of foreign material. Altered excretion by liver, kidney, etc. must be ruled out before we can accept such data as evidence of "blockade."

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## STUDIES ON VITAL STAINING

### III. THE SIMULTANEOUS INGESTION OF TWO DYESTUFFS BY PHAGOCYTES. THE QUESTION OF "BLOCKADE OF THE RETICULO-ENDOTHELIAL SYSTEM"

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#### INTRODUCTION

In previous articles of this series (1) it has been shown that brilliant vital red is relatively non toxic to dogs when given intravenously in rather large amounts. The dye is removed from the plasma, and prior to its gradual elimination from the body is stored in various tissues, mainly in the large groups of phagocytic cells sometimes spoken of collectively as the "reticulo-endothelial system." It was shown that the dye passes from plasma to tissues for many hours, but eventually an equilibrium point is approached, and from this time on dye leaves the circulation very slowly. This virtual cessation of phagocytic activity may appear long before all of the dye has left the circulating plasma, and this is true especially when large amounts of dye are injected. It is in these very cases that the tissue phagocytes themselves are most deeply stained. We concluded that the cessation in phagocytic activity is the result of the partial filling of these cells with dye, but it was possible to show that on injecting more dye the phagocytes will at once resume their activity, and for a time dye leaves the circulation rather rapidly. Quite evidently this injection simply disturbed the balance between dye in plasma and dye in tissues, and the balance could not be restored until part of the newly injected dye had passed from plasma to tissues. This state of balance illustrates a curious form of inactivity on the part of living cells. This inactivity is clearly associated with the presence of dye in the tissues. This is not an inhibition or "blockade" in the ordinary sense of the word, for if we

disturb the equilibrium by injecting more dye we find that the dye-laden cells show a surprising ability to take up the newly offered dye. The evidence indicates that the phagocytic response toward new dye may equal that of normal unstained tissues.

It is of great interest to investigate the reactions of these tissues toward the introduction of a second dyestuff—for analytical convenience a blue dye. We may hope to ascertain whether the presence of the red dye in the tissues will inhibit the entrance of the blue one and whether the final partition of the blue one between plasma and tissues will be influenced by the presence of the red dye in the body. Such studies involve the existence of what we might term "cross inhibition," and it is this form of inhibition to which most workers refer when they speak of "blockade." We may apply the term "specific inhibition" to the concept that tissues laden with one particular dye are so affected that they take up this particular dye not at all or with distinct reluctance. In our own studies such "specific" inhibition was clearly demonstrable only in the sense of an equilibrium between dye in tissues and in plasma. It was impossible to demonstrate "specific" inhibition in the larger sense which involves reluctance of tissues to take up subsequent offerings of this particular dye.

The experiments about to be reported deal with the question of "cross" inhibition, and for purposes of investigation we have injected aqueous solutions of brilliant vital red and of Niagara sky blue. These two dyes belong to the same general class. With the exception of color both have many physical properties in common. Both have certain colloidal properties. In the matter of diffusibility they are rather closely similar. The general similarity in chemical constitution is shown by the structural formulae given below. From these numerous points of similarity one might anticipate finding many points of similarity in physiological behavior also, and we need not be surprised that the two dyes may be demonstrated in the same cells, either side by side in granular form, or, under certain circumstances fused intimately to form purple granules within the phagocytes (2). Here there can be no question that the same group of cells is responsible for removing much of the dye from the plasma—a questionable assumption when widely differing substances are used in the study of "block-

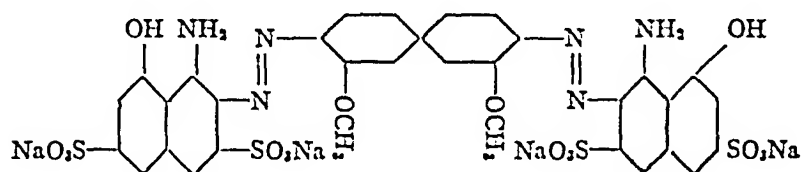
ade." Highly colloidal dyestuffs such as brilliant vital red and Niagara sky blue have much to recommend them in studies of the sort here reported. The relatively slow elimination from the blood stream makes it easy to follow the process by quantitative methods. No doubt the desirability of such studies with dyes so similar in their general properties has occurred to many, but without the spectrophotometric method of analysis (3), it is well nigh impossible to make analysis of the purple plasma which results from the admixture of the two dyes.

The experiments to follow give no evidence to show that the presence of one dye within the tissues influences in any manner the ability of the cells to take up and store another, and this is true regardless of whether the two dyes are given simultaneously or in sequence. Some of the dogs were vitally stained with repeated daily injections of brilliant vital red, after which a measured quantity of Niagara sky blue was injected, and the concentration of this dye in the blood stream determined over a period of several days, and it was found that it left the blood stream at almost identically the normal control rate. In other experiments the two dyes were injected simultaneously, and here again it will be shown that within limits of experimental error the tissues take up the blue dye at the normal rate, and this despite the fact that they were engaged at the same time in taking up the red dye also. Furthermore in the course of 72-96 hours the blue dye is leaving the blood stream very slowly—evidence of a state of equilibrium. The amount of blue dye now in circulation is almost identical with that observed in control experiments where no red dye was given. This shows that the final partition ratio between plasma and tissue is not influenced by the fact that red dye is present also. Presumably in these experiments the red dye is also distributed between plasma and tissues in a manner normally characteristic for that dye. Unfortunately the absorption curves of the two dyes are such that quantitative spectroscopic measurement of the red dye in the purple plasma is not so accurate as in the case of the blue one, and we found it impractical to follow the elimination of the red dye, though attempts along this line have shown that there is certainly no gross deviation from normal in the elimination of the latter.

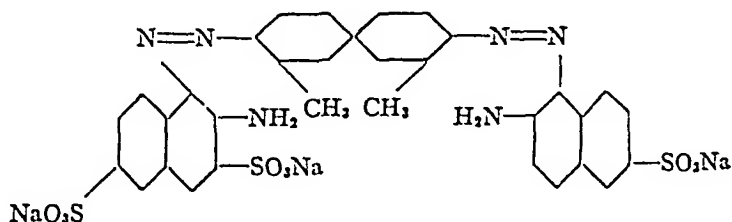


### Methods

Normal adult dogs maintained on a mixed diet were used. As in the previous experiments, feeding hours were so arranged that the plasma would show no lipemia during the hours when samples were taken for dye analysis. The two dyes used, brilliant vital red and Niagara sky blue, were obtained from the National Aniline and Chemical Company. The trade names for these dyes are those given them by that company. It is said that brilliant vital red corresponds chemically to brilliant congo R, rouge congo brillante R, brillantdianilrot R and azidin-scharlach R of other manufacturers. It is Schultz no. 370 and chemically is 1 mol of tolidine combined with 1 mol of  $\beta$  naphthylamine, 3, 6, disulphonic acid and 1 mol of  $\beta$  naphthylamine, 6 monosulphonic acid. Niagara sky blue corresponds to Schultz no. 426 and chemically is 1 mol of dianisidine combined with 2 mols of H acid. It thus corresponds to dyes marketed by various firms under the names of diaminreinblau, bleu pur diamine, dianiblaue H 6 G, oxaminreinblau 5 B, direktblau R B A, etc. The structural formulae are thought to be:



*Niagara Sky Blue*



*Brilliant Vital Red*

For purposes of injection these dyes were made up in a two per cent aqueous solution.

The blood samples were collected in well-vaselined "record" syringes and every precaution was taken to avoid hemolysis. The samples of blood were run into 15 cc. graduated hematocrit tubes containing 2 cc. of a 1.6 per cent solution of sodium oxalate to prevent clotting. After centrifugalization the amount of oxalated plasma in the tube was read off, thus enabling one to correct for the amount of oxalate solution present. The amount of dye in the plasma was determined by means of the spectrophotometer by a method (3) which allows quantitative analysis of each dye even when the two dyes are present together in the plasma.

## EXPERIMENTAL

The data here reported were obtained from similar experiments carried out on 4 different dogs. The individual protocols are presented separately (experiments 45-48 incl.). Table 31 shows the results obtained in the individual cases. One of the experiments (experiment 46) was incomplete, and for this reason it was not used in making up the averages which are shown graphically in Charts 3A and 3B. For ease in presentation we shall refer chiefly to these curves where the progress of dye elimination can be seen at a glance. A careful study of the individual experiments shows minor variations, but all are alike in all essential respects.

*Experiment 45.* Dog 25-29. Male, Collie, 22 kg.

January 18, 1927. Twenty-four cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. At various intervals samples of blood were removed for analysis.

February 13, 1927. An intravenous injection consisting of 24 cc. of 2 per cent Niagara sky blue and 24 cc. of brilliant vital red was followed over a period of 4 days by frequent analysis of the plasma for Niagara sky blue. A sample of plasma taken immediately before making the double dye injection showed that the plasma had become free of the dye injected 3 weeks previously.

May 1 to 9th inclusive, daily injections of 24 cc. of a 2 per cent solution of brilliant vital red.

May 11. Twenty-four cubic centimeters of a 2 per cent solution of Niagara sky blue injected while the plasma still contained large quantities of brilliant vital red (about 400 mg. dye per liter plasma). Samples of blood taken during the next 4 days were analyzed for their content of Niagara sky blue.

The results of all of these analyses are shown in Table 31.

*Experiment 46.* Dog 25-35, Male, Setter, 18 kg.

March 30, 1927. Twenty cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. During the next 4 days samples of plasma were collected for dye analysis.

May 4, 1927. Plasma found to be free of dye. Intravenous injection of 20 cc. of a 2 per cent solution of brilliant vital red together with 20. cc. of a 2 per cent solution of Niagara sky blue. Samples were taken for analysis at intervals during the next 4 days.

The results of the analyses are shown in Table 31.

*Experiment 47.* Dog 25-16. Airedale, 19 kg.

January 19, 1927. Intravenous injection of 20 cc. of a 2 per cent solution of Niagara sky blue. The concentration of dye in the plasma was determined at intervals during the next 4 days.

February 11, 1927. The plasma was free of dye. Intravenous injection consisting of 20 cc. of 2 per cent brilliant vital red along with 20 cc. of 2 per cent Niagara sky blue. Analyses of Niagara sky blue in the plasma at intervals during the next 4 days.

May 3 to 11th. Daily intravenous injections of 20 cc. of a 2 per cent solution of brilliant vital red.

TABLE 31

	Mg. Niagara sky blue per liter plasma after					
	5 min.	1 hour	6 hours	24 hours	48 hours	96 hours
Experiment 45						
Niagara sky blue alone.....	360	240	170	46	32	22
Niagara sky blue after chronic staining with brilliant vital red.....	310	210	130	43	30	16
Niagara sky blue in presence of brilliant vital red simultaneously injected.....	290	210	115	50	43	30
Experiment 46						
Niagara sky blue alone.....	340	190	81	36	17	10
Niagara sky blue in presence of brilliant vital red simultaneously injected.....	335	205	79	39	20	14
Experiment 47						
Niagara sky blue alone.....	330	195	90	44	18	12
Niagara sky blue after chronic staining with brilliant vital red.....	320	190	94	30	14	9
Niagara sky blue in presence of brilliant vital red simultaneously injected.....	300	192	99	45	23	15
Experiment 48						
Niagara sky blue alone.....	320	175	75	40	25	12
Niagara sky blue after chronic staining with brilliant vital red.....	275	180	91	59	23	10
Niagara sky blue in presence of brilliant vital red simultaneously injected.....	303	175	91	65	38	11

May 13. The dog is deeply stained and the plasma contains large amounts of the red dye (about 390 mg. dye per liter plasma). Twenty cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. Analyses were made at intervals during the next 4 days to determine the amount of the blue dye in the plasma.

The results of all the analyses are recorded in Table 31.

*Experiment 48.* Dog 24-74. Female, Shepherd, 28 kg.

January 20, 1927. Intravenous injection of 30 cc. of a 2 per cent solution of Niagara sky blue. The concentration of dye in the plasma was determined at intervals during the next 4 days.

February 15, 1927. The plasma was free of dye. Intravenous injection consisting of 30 cc. of a 2 per cent solution of brilliant vital red along with 30 cc.

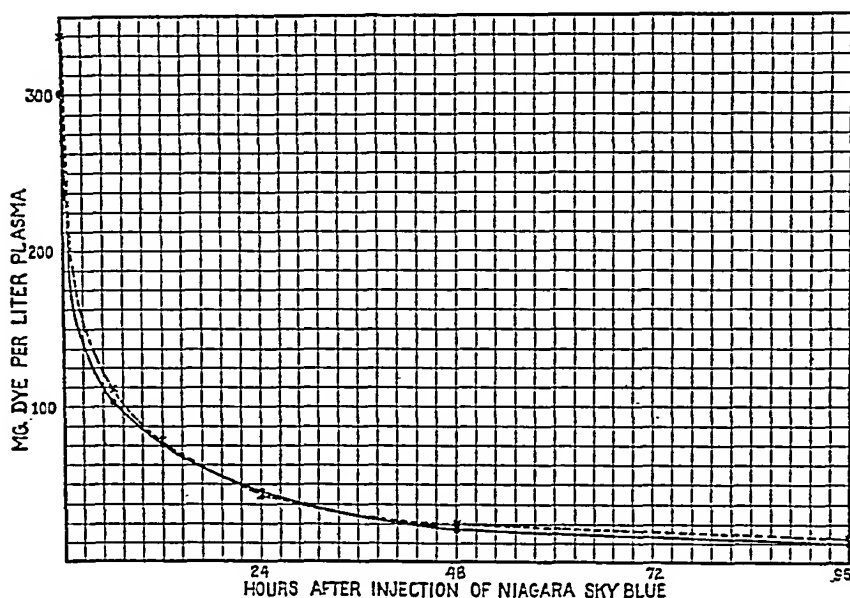


CHART 3A. Concentration of Niagara sky blue (average of 3 dogs). Interrupted line = unstained dogs. Continuous line = dogs previously stained with brilliant vital red.

of a 2 per cent solution of Niagara sky blue. Analyses of Niagara sky blue in the plasma at intervals during the next 4 days.

April 30 to May 8th inclusive, daily injections consisting of 30 cc. of 2 per cent solution of brilliant vital red.

May 10. The dog is deeply stained and the plasma contains large amounts of dye (about 320 mg. dye per liter plasma). Thirty cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. Analyses were made at intervals during the next 4 days to determine how rapidly the Niagara sky blue left the blood stream.

The results of all of the analyses are shown in Table 31.

Samples of centrifuged blood taken 5 minutes after an intravenous injection of brilliant vital red show plasma which is of a red color. If the dye present be measured colorimetrically it will be found that the amount present is directly proportional to the amount injected and inversely proportional to the total plasma volume of the animal. Similarly, an injection of Niagara sky blue imparts a bluish color to the plasma. Like brilliant vital red, this dye leaves the blood stream,

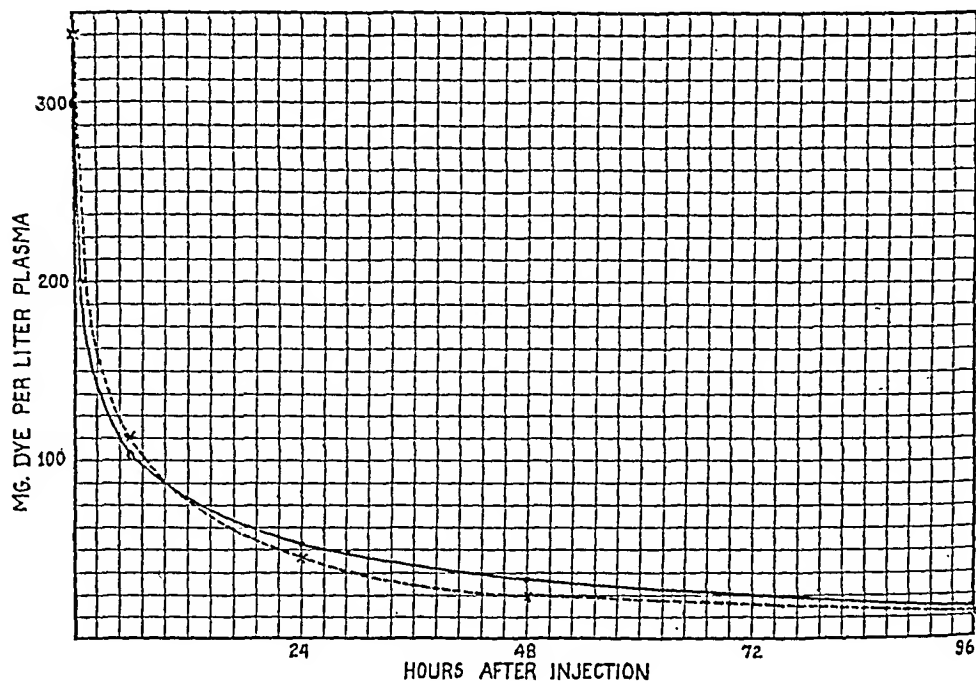


CHART 3B. Concentration of Niagara sky blue (average of 3 dogs). Interrupted line = Niagara sky blue injected alone. Continuous line = Niagara sky blue injected simultaneously with brilliant vital red.

and within 48 hours most of it is lodged in specialized phagocytic cells in various parts of the body. The rapidity with which each dye leaves the blood stream is somewhat different for each dye, and there are small individual differences in animals of the same species. With a given dye and under normal conditions the form of this curve is remarkably constant for a given animal.

We have given a series of four dogs an initial standardizing intravenous injection of Niagara sky blue. In each case the concentration

of dye in the plasma was measured five minutes later and again at intervals during the next 72 hours. A number of weeks were then allowed to elapse in order that the body might rid itself of the dye and be in a position to react normally once more. Each animal now received eight large daily injections of a two per cent solution of brilliant vital red. The tissues became progressively more red until staining was finally quite intense. To ascertain whether this red staining of the tissues reduces the ability of the latter to take up the blue dye from the plasma we then made an injection of the latter. We note from Chart 3A that, as in the original control experiment, the blue dye rises abruptly to a level of about 300 mg. per liter. From this point on until the end of the experiment 96 hours later the two curves are almost identical, showing quite unmistakably that these red-stained tissues will take up the blue dye just as effectively and as rapidly as do unstained tissues.

Some critic may wish to urge the point that some original phagocytic inhibition may have worn off before the test dose of blue dye was given. Indeed, it is held by many that dye-laden phagocytes may multiply by cell division or that new phagocytes may form from connective tissues with great rapidity and that these newly formed cells may take over the lost function of the stained cells, and thereby compensate for their deficiency. This point of view is thoroughly discussed by Aschoff (4). We must agree that some such increase in cells does occur when heavy injections are made repeatedly. We have also confirmed older observations that the individual phagocytes increase in size as they take up dye. But these data, presented in a paper to follow (2), include also some observations on double staining with these two dyes. It can be shown that when the two dyes are given in successive periods, the phagocytes contain granules of red and granules of blue dye, side by side. Very rarely indeed do we see cells which contain only one type of dye as we might expect if great numbers of new phagocytes were formed in the period of observation. The separate storage of materials in different cells reported by others need not signify that newly formed cells have been formed to take up the second substance; more likely is the view that the two substances used differed so widely that they simply found storage in cells which differed somewhat in type or in accessibility to the circulating blood.

We feel that purely morphological studies of these questions give only qualitative notions about the importance of cell multiplication in compensating for a hypothetical blockade. We are presenting certain physiological data which bear upon this point. The experiments were performed on the same dogs which we used in the experiments presented above. This second part of each experiment consisted in an effort to determine whether the tissues would take up Niagara sky blue at the normal rate if they were simultaneously busied with the phagocytosis of brilliant vital red. The animals were free of dye at the beginning of the experiment, but when the two dyes were mixed and injected we note that they gradually disappear from the plasma and become lodged largely within the tissues. It was thought that as the dyes accumulate together within the cells we might find a mutual antagonism—in other words, some evidence of failure of the cells to perform the double task of removing two dyes, each at its own normal speed. The fact that no such delay was observed is especially significant in these acute experiments. If phagocytic inhibition is to be observed at all we might expect surely to see it in these early stages of staining before cell multiplication could compensate for the filling of the cells with dye. If cell multiplication is responsible for the maintenance of phagocytic efficiency of the tissues we must be prepared to admit that the response is almost instantaneous—that there is little or no lag between incipient inhibition and compensatory multiplication and enlargement of cells.

In carrying out the experiments of which we have just spoken the two dyes were mixed in equal amounts and the mixture was injected into the blood stream. By means of the spectrophotometer we were able to measure the amount of each dye in the purple plasma which resulted. Samples of plasma were taken at intervals over a 72 hour period. From a technical point of view it is rather difficult to make precise measurements of brilliant vital red in this purple plasma, but the measurement of Niagara sky blue is much more easy and exact, for there is very little overlapping of the absorption bands at the red end of the spectrum where the blue dye must be measured. Furthermore the slight jaundice which sometimes develops does not interfere with the measurement of the blue dye as much as with the measurement of the red one. The concentration of the blue dye is shown in

Table 31 and graphically in Chart 3B. In this chart is also shown the normal control curve obtained some weeks previously by means of a simple injection of the blue dye alone. The two curves are almost identical throughout. The very slight retardation in elimination in the presence of the red dye is probably within the limits of experimental error. Like the experiments cited above, this experiment shows that a dye will pass from plasma into tissues at its own normal rate regardless of the presence of another dye. It is quite apparent that the tissues take up each dye in an independent manner.

#### DISCUSSION

We have already cited articles in which the reader will find discussions of the literature of "blockade" of the reticulo-endothelial system. It requires great generosity to admit much of the evidence which has been advanced in favor of the concept. We cannot but feel that much of the data is totally irrelevant. Changes in immune reactions, in fat metabolism, in pigment metabolism, and so forth, may well be associated with the injection of some foreign substance or with the use of some poison, and by some this is offered as evidence that the reticulo-endothelial system is blocked. Curiously enough this same evidence is offered by others to prove that the reticulo-endothelial system is concerned with immune reactions, fat or pigment metabolism. Obviously neither contention is built on firm foundation. Such rude procedures as these must cause great disturbances in the delicately regulated organism. Important changes in parenchymatous organs, changes in excretory activity of various organs are important considerations, and in our enthusiasm for the reticulo-endothelial system we must not neglect these other factors.

The rate at which dye leaves the blood stream depends upon excretion by liver and kidneys as well as upon the activity of the body phagocytes, nor, as we have shown, is it possible to disregard excretory activity by assuming it to be proportional to dosage. Such excretory activity must be measured carefully before we can assign to the phagocytes their proper importance in freeing the blood stream of dye, or of other materials. It is astonishing to see how generally this elementary precaution has been neglected. We have made some



progress in a study of the rate at which the liver excretes brilliant vital red, and certain of the data have been presented in the preceding paper. Unfortunately, Niagara sky blue is partially decolorized on reaching the bile, and for this reason it has not been possible to follow its elimination by the liver. The experiments presented above show that this dye leaves the blood stream at a normal rate, even though the body contains large amounts of red dye, and we have offered this as evidence that such red-stained tissues will take up the blue dye at a normal rate. Had the experiment turned out differently, and had we observed undue retention of the blue dye in the plasma, we would have been faced with the necessity of ascertaining whether defective phagocytosis or defective liver elimination was to blame, but since elimination is observed to be quite normal we do not need to postulate defective liver excretion. Indeed, if this could be shown to exist in this case, we would be forced to conclude that the red-stained tissues take up the blue dye *more* readily than normal, but there is no evidence to indicate that this is true, though this rather remote theoretical possibility must be kept in mind.

All things considered, we feel that brilliant vital red and Niagara sky blue are particularly well suited to studies on "blockade." They are not readily precipitated from solution by salts common in plasma or other body fluids and this is more than can be said of many substances which have been employed by others. The carbon of India ink is precipitated almost the moment it comes into contact with the plasma, and the rate at which these particles are filtered out of circulation gives us no clue about the activity of the phagocytic system. This same objection may be raised against the use of colloidal metals against the use of emulsions of fats and other substances.

The dyes we have used are free of these objections and we may confine our attention to storage by the tissues and to excretion from the body. These dyes are so much alike that they are taken up and stored within the same identical cells. With double staining experiments it is very rare to find cells which contain only one of the two dyes injected (2). It is quite otherwise when substances of widely different sort are used in combination. It is well known that the carbon particles of India ink do not pass readily through the capillary

walls. They soon become lodged within the phagocytes which are most directly related to the blood streams. We see them particularly in the Kupffer cells of the liver and in certain cells of the spleen. The enormous group of phagocytes in lymph nodes and in the connective tissues generally receive little or none of the carbon until a long period of time has elapsed. With such a substance there is no possibility of "blocking" more than a small part of the reticulo-endothelial system. One might well expect a normal response toward dyes and toward many other substances which readily reach cells which never come in contact with the ink. From such considerations we see the need of microscopic studies as controls in order that we may know at all times just which cells are concerned in any given process.

#### SUMMARY

When large amounts of brilliant vital red are injected into the blood stream of dogs, the dye is gradually removed from circulation, and most of it is deposited in numerous phagocytic cells which are scattered throughout various organs and tissues. The dye occurs largely in the form of tiny red granules crowded together in the cytoplasm of these cells.

If Niagara sky blue, a closely related dyestuff, is injected, it too is taken up and stored in these same cells. It is shown that the presence of red dye in the tissues does not inhibit the cells from taking up the blue one.

The normal ability of the phagocytes to take up Niagara sky blue is observed also when this dye is injected *simultaneously* with brilliant vital red. This normal response toward the blue dye is seen even though the phagocytes are busied at the same time in the process of engulfing and storing the red dye.

These experiments show that it is difficult if not impossible to "block" the cells with one dye so that their ability to take up another is even slightly impaired.

The two dyes employed in these studies are shown to be particularly suitable for experiments of the sort here reported.

The author wishes to express appreciation to Miss E. Fairman for technical assistance in making many of the color analyses.

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# CUTANEOUS REACTIONS WITH CULTURE FILTRATES OF THE COLON TYPHOID TYPE

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## HISTORICAL REVIEW

It has long been known that products of the typhoid bacillus induce a skin reaction. McKendrick (1) in 1923 carefully reviewed the early publications on the subject. In 1914 Gay (2) and his co-workers were able to demonstrate with the use of their so-called "typhoidin test" cutaneous manifestations, particularly in cases of recovery from typhoid fever irrespective of the antecedent date of the disease. The same reaction occurs only in relatively few instances in individuals who give no history of typhoid fever or typhoid vaccination. The original products of Gay were made in a manner similar to that used in the production of tuberculin and therefore contained many unknown substances which might greatly influence the results. Force and Stevens (3) improved the test by the use of an alcohol and ether dried typhoidin, precipitated from plain instead of from glycerol broth. By the use of this product Gay and Lamb (4) obtained improved results. However, considerable controversy has arisen as to the specificity of the test, since it was established by Nichols (5) that in individuals vaccinated against typhoid fever, 66 per cent reacted to paratyphoidin A and no more than 75 per cent to typhoidin. Nichols regarded a positive reaction as evidence of typhoid protein sensitization, which he thought was not synonymous with true disease immunity. Nichols and Hitchens (6) later concluded that several factors operate and should be considered in the interpretation of the results. In these they included allergy, toxicity, and non-specific factors such as personal idiosyncrasy. Similarly the work of Meyer and Christiansen (7) militated against the conclusion reached by Gay and his co-workers. Austrian and Bloomfield (8) also stated that normal persons and those who had had typhoid fever reacted in cutaneous tests with typhoidin; they, however, emphasized the value of the test when negative. Thompson (8) in 1921, applied three loopfuls of killed cultures to a clean and scarified area of the skin and noted that in a positive case there appeared to be continuance of the "red reaction" into the fourth day and commented on the possible value of the test for the detection of carriers.

McKendrick (1. c.) using standardized suspensions of typhoid bacilli with reference to agglutinability recorded that the procedure used by him constantly gave

positive reactions both in patients suffering from enteric fever and in chronic carriers. The test appeared to be highly specific. Early in convalescence the skin test became negative. Fleisher and Tierney (9) in 1926 used broth culture filtrates in 1:500 dilution and noted variability in the normal controls. In cases of the fever, the reaction was markedly diminished as compared with the weakly reacting normals or was negative. Early in the disease the reaction might be distinct, but later it became negative. Howell and Corrigan (10) also used broth culture filtrates and suggested that the reaction is allergic in nature, a positive reaction indicating immunity since those immunized or those with a history of typhoid fever, and those in the course of the disease tended to react positively in most instances; whereas only 40 per cent of other adults and 4 per cent of children gave a positive reaction.

In 1927 Ecker and Rimington (11) showed that synthetic medium culture filtrates of the aertrycke type of paratyphoid bacilli, concentrated in vacuo and dialyzed, when injected into the skin of normal (non-sensitized) rabbits produced a raised edematous and reddened area. After decomposition of a lead precipitate of the same filtrate material by means of  $(\text{NH}_4)_2\text{SO}_4$  and dialysis, a marked reaction was also obtained by a similar injection, the wheal being about 1.5 cm. in diameter within 24 hours. The center was about 4 mm. in diameter, elevated and white, and the surrounding areas was hyperemic. In about 4 days the central area became necrotic and the hyperemia subsided. The original filtrate (not inoculated, concentrated, or dialyzed) failed to give a reaction. The concentrated products gave certain color reactions which are usually associated with the presence of carbohydrates. Small quantities of nitrogen were also obtained (0.3 to 0.4 per cent). So far as we know this observation is the first one correlating bacterial carbohydrates with toxicity.

Up to 1928 the demonstration of skin reactivity with filtrates had been accomplished only by intracutaneous injections. Shwartzman (12), however, in that year demonstrated a new phenomenon. He observed that following an intracutaneous injection of the filtrate (tryptic digest broth) of the typhoid bacillus an intravenous injection caused a hemorrhagic area to appear at the point of the original intracutaneous injection. He has produced the phenomenon with filtrates of a large number of organisms. The reactions were fully developed after 4 or 5 hours following the intravenous injection of the filtrate. This author was able to neutralize the filtrate to be used for the intravenous injection with the specific immune antityphoid serum.

In his early work Shwartzman used a tryptic digest broth but to avoid extraneous materials he later used growths on veal infusion agar, washing the organisms from the surface with 0.9 per cent saline. The centrifuged suspensions were then passed through Berkefeld "N" candles, but this filtrate gave very little reaction intracutaneously. He further noted that the filtrate was inactivated only by autoclaving. Dilutions as high as 1:48 when injected into the skin prepared it for the "Shwartzman phenomenon." Two months after the publication of Shwartz-

man, Hanger, Jr., (13) reported that when animals showing a positive skin test to filtrates of *B. leprosepticum* received an intravenous injection of the filtrate, after several hours obliteration did not occur but an intensification of the local reaction was found, and the site of inoculation often showed blue discoloration with areas of petechial hemorrhages. In most instances, an extensive local necrosis occurred. The author stated that he has never observed the phenomenon except among rabbits naturally allergic or rendered allergic by a previous infection. The reaction was proportional to the allergy of the animal to the filtrate used and appeared to be non-specific. He further stated that it is rather obscure why the filtrate diluted in the blood is more destructive to endothelium than the same filtrate concentrated locally. The intensified reaction described by Hanger appears to be the same type of reaction first described by Shwartzman. In a previous paper (same year) Hanger (13) showed that rabbits are often naturally allergic to filtrates of many gram negative bacteria but in this report no mention was made of the intensified (hemorrhagic) reaction.

In our work we have attempted to procure as pure products as possible in order to avoid extraneous materials which might introduce confusion. The method described by Ecker and Rimington was used to produce the pure filtrates. We have studied the effect of heat and light on the production of a skin reaction with the concentrated filtrates; and the possibility of producing an immunity to these filtrates by various methods: (a) intravenously, (b) intravenously with massive doses of the concentrated filtrates and (c) intracutaneously. Attempts were made to study and to explain the mechanisms involved when the toxic filtrates are mixed with the homologous antisera and introduced into the skin. We watched carefully the cutaneous reactions obtained in the immunized animals. To determine whether or not specificity existed "crossing over" experiments were made *in vivo*. Experiments were also made to observe the effect of normal human and convalescent typhoid sera on the filtrates intracutaneously injected. Attempts were made to note the relation of the Shwartzman products to ours, and whether or not filtrates from enterococci would induce the Shwartzman phenomenon in animals prepared with filtrates of the colon-typhoid group of organisms. Incidentally all the sera of the immunized animals were examined for the presence of precipitins.

### *Method*

Seven members of the colon-typhoid group of organisms, namely, 1 *B. typhosus*, 1 *B. paratyphosus* A, 2 *B. paratyphosus* (aertrycke type), 1 *B. enteritidis*, 1 *B.*

paratyphosus B. (Schottmüller), and 1 B. coli were employed. The organisms were plated out and the smooth colonies—known to be the best producers of toxic filtrates (Ecker)—isolated in all cases. They were then made to grow by repeated transplantations on the medium of Cahn-Bonner (See Ecker and Rimington, l. c.). After five days of growth the pure cultures were centrifuged and passed through Berkefeld "N" candles. The bacteria-free filtrates were then concentrated in vacuo and dialyzed in collodion sacs against running distilled water until salt free with  $\text{AgNO}_3$ .

Strict asepsis was observed. The concentrated and dialyzed filtrates were made isotonic with 0.9% NaCl and ampouled. The ampoules were then heated at 60°C. for one hour and tested for sterility.

As controls the same medium (to which *no* organisms were added) was filtered, concentrated, dialyzed, and made isotonic. As a further check the condensate and dialysate were treated in a like manner. Finally in a large series of our rabbits sterile 0.9% NaCl was injected intracutaneously. The controls were negative in all cases.

The toxicity of the filtrates was determined by intravenous injections of rabbits. The typical picture described by Ecker and others was demonstrated. All the rabbits used weighed from 2000 to 3000 gms. The 17 animals used for immunization purposes were given injections over a period of ten weeks as follows:

(1) Intravenous injections with increasing doses of the dilute and concentrated filtrates starting with 0.3 of one cc. (dilute) and ending with 1.5 cc. of the concentrated filtrates.

(2) Intravenous injections with massive doses of the concentrated filtrate (4 injections of 5 cc. each over a period of 20 days).

(3) Intracutaneous injections of about 11 cc. given in 0.2 cc., doses totaling approximately 55 injections over a period of 10 weeks.

All the rabbits were bled by cardiac puncture, 11 weeks after the beginning of the immunization. Prior to the puncture food was withheld for at least 24 hours so that clear sera for precipitin tests would be obtained.

Finally all the immunized animals were given intracutaneous injections of the homologous and heterologous filtrates.

Where attempts were made to neutralize the toxic filtrates by the homologous immune sera the mixtures were incubated for 2 hours prior to the intracutaneous injection.

All the animals used for the intracutaneous injections were depilated on both flanks with a mixture of barium sulphide, zinc oxide, and starch to avoid irritation. The animals in most cases were injected 24 hours after depilation.

#### EXPERIMENTAL

(I) *Effect of Heat and Light on the Filtrates.*—The filtrates were exposed to heat and ultraviolet light to observe whether or not their

toxicity was diminished. The size and character of the wheals were noted and tabulated. The results of the experiments are given in Table I.

From this table it is seen that in all cases the controls were positive, the wheals varying in size in different animals. It will be further

TABLE I  
*Showing the Effect of Heat and Ultraviolet Light on the Toxicity of the Filtrates*

Filtrates of	Control reaction	Heated at 100°C. for 20 minutes	Auto- claved at 15 lbs. for 15 min.	Ultraviolet light "C" carbon 15 minutes (50 cm.)	Ultra- violet light "C" carbon for 30 minutes
<i>B. typhosus</i>	Wheal 12 x 12 mm. red +	Wheal 11 x 10 mm. red +	—	Wheal 12 x 15 mm. red +	±
<i>B. paratyphosus</i> A	Wheal 14 x 12 mm. red +	Wheal 11 x 9 mm. red +	—	Wheal 10 x 8 mm. red +	—
<i>B. coli</i>	Wheal 17 x 12 mm. red +	Wheal 11 x 9 mm. red +	—	Wheal 10 x 8 mm. red +	—
Aertrycke 185 <i>B.</i> <i>paratyphosus</i>	Wheal 8 x 6 mm. red +	Wheal 7 x 5 mm. red +	—	Wheal 6 x 7 mm. red +	—
( <i>Deli B. enteritidis</i> )	Wheal 15 x 14 mm. red +	Wheal 11 x 18 mm. red +	—	Wheal 13 x 12 mm. red +	±
Aertrycke de Nobele <i>B. paratyphosus</i>	Wheal 12 x 11 mm. red edema- tous +	Wheal 8 x 6 mm. red +	—	Wheal 6 x 6 mm. red +	±
<i>B. paratyphosus</i> B. Schottmüller type	Wheal 10 x 8 mm. elevated red +	Wheal 6 x 5 mm. red ele- vated +	—	Wheal 9 x 8 mm. red +	—

noted that heating at 100°C. for 20 minutes did not destroy completely the toxicity of the filtrates. Autoclaving, however, at 15 lbs. for 15 minutes, destroyed or inhibited the reaction-producing material in all seven filtrates. Ultraviolet light with a "C" carbon (see Perkins and Welch (14)) with an exposure in quartz tubes of 30 minutes at 50 cm. distance using 55 volts and 30 amperes completely



inhibited or destroyed the toxicity in 4 cases. In three cases a questionable reaction developed. Exposure of 15 minutes under the same conditions failed to weaken the potency of the filtrate.

(2) *Effect of Dilution on the Toxicity of the Filtrates.*—The seven concentrated filtrates were diluted, as shown in Table II, with 0.9% NaCl, and 0.2 of one cc. was inoculated intracutaneously. It will be

TABLE II  
*Showing Endpoint by Dilution of Concentrated and Dialyzed Filtrates*

Filtrates of	Dilutions					
	0	1:2	1:4	1:8	1:10	1:16
B. typhosus.....	+	+	+	—	—	—
B. paratyphosus A.....	++	++	+	±	—	—
B. coli.....	++	++	+	±	—	—
Aertrycke 185 } B. paratyphosus }	++	++	++	+	+	±
B. enteritidis (Deli).....	++	++	++	+	+	±
Aertrycke de Nobele } B. paratyphosus }	++	++	+	±	—	—
B. paratyphosus B. } Schottmüller type }	++	++	++	+	+	+

++ large, red and edematous.

+ definite red area.

± Small pale, pink area.

— No reaction.

seen that the loss of ability to produce a skin reaction varied for the different filtrates; the B. paratyphosus Schottmüller, aertrycke 185, and B. enteritidis strains remained positive when diluted 1:16; the B. paratyphosus A, B. coli, and aertrycke de Nobele were positive at 1:8; the B. typhosus strain was positive only in a dilution of 1:4. See Table II.

(3) *Effect of CaCl<sub>2</sub> in 1:500 Dilution. (Kataphylaxis).*—Introduc-

tion of  $\text{CaCl}_2$  in 1:500 dilution in saline which in itself produces no visible reaction in the skin, was found to increase the activity of the filtrates when injected into the same area. The reactions persisted from four to six days but the control reactions with the filtrates usually disappeared in about three days.

(4) *Effect of Freezing on Filtrates*.—Freezing of the filtrates until solid with subsequent gradual thawing indicated a possible method for concentration, inasmuch as the part which melted first caused typical reactions. This method, because of its practical significance, is being studied further in this laboratory.

### *Immunization*

(1) *Size and Character of Wheal before, during, and after Immunization*.—During immunization, by the intracutaneous method, the size and type of the wheals were carefully determined. It was observed that there was a definite decrease in size and character (less red) of the wheals. Originally they measured from two to three cm. in diameter, showing a deep red, edematous area, but as time went on the areas decreased in size until following the final injection (10th) the size of the wheals had diminished to from 5 to 7 mm. in diameter. However, it was also noted that whereas the wheal at first appeared flat, edematous and red, it now became nodular, definitely showing an *indurative* character. In one animal a row of seven nodules (7 injections of 0.2 of one cc. each, the 6th intracutaneous injection) persisted for over one week. Animals that received massive doses of the concentrated filtrates by vein also showed a similar indurative reaction, when they received, after immunization, the homologous filtrates intracutaneously.

In spite of the fact that 2 to 3 cc. of the original filtrate (dilute) caused death of a large number of normal animals, it was found that immunized animals could tolerate massive doses (5 cc.) of the concentrated and dialyzed filtrate. This is interesting since 5 cc. of the concentrated filtrate represent 50 cc. of the original filtrate.

(2) *Precipitin Content of Sera*.—All the sera have been tested for their precipitin content by the ring method in Hektoen tubes. As antigen we used both the concentrated and the original dilute filtrate. On testing the sera from animals that received the filtrate intracutaneously no precipitins were demonstrated.

The sera of animals that received the filtrates by vein in increasing and finally in massive doses all showed precipitating qualities in varying degrees. The strongest reactions were found in the sera of animals that received the filtrate from the typhoid bacillus and from the *B. paratyphosus aertrycke*, type "de Nobele." The other five sera gave moderate but definite rings with the dilute filtrates.

The sera obtained from animals that received massive doses of filtrates from the typhoid bacillus, aertrycke 185, and *B. paratyphosus A.* gave the most marked precipitin reactions. In all cases a three plus reaction was obtained when using both the dilute and the concentrated filtrates as antigen. All the tubes were given at least 20 hours at room temperature before a final reading was taken, and in no instance did salt solution cause a ring to be formed.

(3) *Neutralization of Toxic Filtrates by Normal Human, Convalescent Typhoid and Rabbit Sera.*—We secured three sera from human typhoid cases. Of these one (A) was from a convalescent 5 weeks after the onset of the disease; the second (B), and the third (C) were from cases that gave a negative Widal but from which the organisms had been obtained. The serum from (B) was obtained 4 weeks after the onset of the disease and from (C) during the first week.

Two methods of neutralization were attempted: First, increasing amounts of the sera mixed with our dilute and concentrated filtrates (typhoid) were injected intracutaneously, and second, the sera alone were injected into the skin and 24 hours later, the filtrates into the same areas. Each serum was tested on one animal. In all cases we were unable to demonstrate a neutralization of the toxic effect. The wheals were larger than the controls, deeper red and hard. There was a definitely, indurated area. We injected in these instances the antigen (antibody complexes) into the skin:

Using the same method but now with six normal human sera (two obtained from cases of hypertension) we found that in all cases, except one, the reactions were of the same size and color as the controls. In the one normal serum which gave no reactions with the filtrate we demonstrated agglutinins in a dilution of 1:64, whereas the others were all less than 1:15.

As a further control a rabbit antityphoid serum from which a serum titer 1/640 was obtained and 4 other normal rabbit sera were

tested by the two methods just mentioned. The immune serum definitely increased the size of the wheals when injected with its homologous filtrates and the areas were red, (raised and indurated). When the normal rabbit sera were individually mixed with each of the seven filtrates, we observed definite areas but of a different character than that of the immune rabbit serum causing wheals which were red, flat and edematous, with no induration. It is therefore to be assumed that during the course of the immunization with a whole typhoid culture of the animal, we have also sensitized the animal to the bacterial products. Further studies along this line will be published at a later date.

(4) *Effect of the Anti-Filtrate Sera on the Filtrates.*—Seventeen anti-filtrate sera were employed. Of these seven were prepared by intravenous injections of increasing doses, three by intravenous injections of massive doses, and seven by intracutaneous injections of the concentrated filtrates. All the sera were inactivated at 55°C. for 20 minutes. The sera were mixed with the homologous filtrates in equal portions and incubated for two hours at 37°C. The mixtures of sera and homologous filtrates were injected intracutaneously into a series of rabbits. The results were as follows:

(A) The sera from the animals that received increasing doses of the filtrate intravenously mixed with the concentrated filtrates caused raised indurated and reddened areas.

(B) The three sera obtained from animals that received massive doses mixed intravenously with the concentrated filtrates and injected caused the appearance of red, edematous and indurated wheals.

(C) The sera from the intracutaneously treated rabbits mixed with the filtrates caused slightly edematous, red areas but no indurations were noted. (These sera had no precipitins.)

Two of these animals (from A) were then given 5 cc. of the concentrated filtrates (*B. coli* and *B. paratyphosus aertrycke*) by vein to produce a Shwartzman reaction. In four to five hours after the injection of the filtrate a marked hemorrhagic, blue-black area appeared at the point of former injections.

(5) *Specificity by the "Shwartzman Phenomenon" in the Group.*—Each of seven rabbits received the seven filtrates intracutaneously in the usual 0.2 of one cc. doses. The initial reactions were about 10 to

TABLE III

*Showing the Effect of the Intravenous Injection of Seven Different Filtrates in Rabbits Previously Injected with all the Filtrates Intracutaneously (Shwartzman Technic)*

Intracutaneous injections with 0.2 cc. of filtrates of the following filtrates										
Rab- bit No.	Filtrate injected intravenously 24 hrs. after intracutaneous injection	Dose in cc.	B. typhosus	B. paraty. A.	B. coli	B. paraty. 185. Aertrycke type	B. enteritidis	Aer. de Nobele B. paraty. B.	Schottmüller B. paraty. B.	
3	B. typhosus	5	—	12 x 12 mm. dark red	4 x 7 mm. red	—	4 x 7 mm. blue hemor- rhagic	—	—	
8	B. paraty. A.	4	Original area dk. red	Original area dk. red	Original area dk. red	Original area dk. red	Original area dk. red	Original area —blue in color	—	
7	B. coli	4.5	5 x 5 mm. blue	5 x 5 mm. blue	25 x 15 mm. blue with dk. red center	—	Original area darker red	5 x 5 mm. blue	—	
2	B. paraty. B. aer. 185	5	4 x 5 mm. red	—	4 x 5 mm. red	—	4 x 5 mm. red	—	—	
5	B. enteritidis (Deli.)	5	8 x 12 mm. purple (hemor- rhagic)	—	12 x 12 mm. Purple, Intracuta- neous hem- orrhages	Original area pale blue	12 x 14 mm. purple severe cutaneous hemor- rhages	—	4 x 4 mm. Purple and markedly hemor- rhagic	

1	B. paraty. B. acr. de Nobele	5	—	8 x 8 mm. blue mod- erately hemor- rhagic	5 x 5 mm. Pale blue mildly hemor- rhagic	5 x 5 mm. Pale Blue	—	—	—
6	B. paraty. B. Schott- müller	3	—	—	—	—	—	—	—

12 mm. in diameter. They were edematous, flat and red. Twenty-four hours later each animal received one of the seven filtrates (3 to 5 cc. concentrated) by vein. The results are tabulated in Table III.

From this table it is apparent that the filtrate from *B. paratyphosus* Schottmüller type has failed to produce a reaction. This is probably due to the fact that only 3 cc. were given by vein or possibly to variability of the animal. When five cc. were used, the reaction appeared in all cases. The other filtrates have caused more or less severe reactions, the homologous areas being often markedly hemorrhagic. The experiment shows that although the homologous filtrates usually causes a typical Shwartzman reaction to appear, heterologous filtrates of the same group will also in some cases produce the phenomenon. We were primarily interested in comparing our filtrates with those of Dr. Shwartzman, who kindly sent us some of his *B. typhosus* filtrates. To test the relationship we injected into the skin of two animals, 0.2 of one cc. of Shwartzman's typhoid filtrate and 0.2 of one cc. of our own typhoid filtrate. After a period of 24 hours, our filtrate showed a definite red wheal; whereas Shwartzman's filtrate showed only a pale pink area. One animal was now given 5 cc. of the Shwartzman product and the other, 5 cc. of our filtrate. All four of the intracutaneous reactions had changed character within 5 hours. The area now appeared hemorrhagic, measuring 10 x 10 mm. in diameter. It seems therefore since his product has the capacity to prepare the skin for our product and, vice versa, our filtrate prepares the skin for his product, that we are without a doubt dealing with the same factor or factors. The only difference between the two is that with our own filtrates we always obtain a red, edematous reaction with the intracutaneous injection. This we now know is due to concentration of the filtrates.

As controls we gave the second injection subcutaneously but no hemorrhagic areas developed.

Similarly broth culture filtrates from three serologically different types of enterococci of Welch (15) were injected intravenously into a series of three animals whose skin had been prepared by all our seven filtrates. No hemorrhagic areas developed, definitely demonstrating at least a group specificity. Broth alone or saline containing 1 per cent nucleic acid were also injected into prepared animals with negative results.

## DISCUSSION

Considerable controversy has arisen as to the value of skin tests in the course of typhoid fever. The methods of preparation of the reacting agents have been many and varied, and the mechanism involved little understood. In most instances filtrates of complex media cultures were employed, rather than suspensions of the organisms or fractions thereof. It must be assumed that during the course of the disease and probably also in the carrier state bacterial allergy exists, and little is known concerning the development and persistence of allergic state in this disease. That the reactions may be allergic is not a new conception.

In the present study we paid careful attention to the production of a bacterial colloid, free from all possible extraneous materials. We showed that with the concentrated synthetic medium culture filtrates of Ecker and Rimington, erythema and edema can be readily produced in the rabbit's skin; while the original, unconcentrated filtrate, rarely produces an inflammation. Immunization experiments by the intracutaneous and the intravenous route definitely demonstrated a reduction in the reaction but obliteration was not noted and at the same time an indurative reaction was observed. The mechanism therefore is one of antigen-antibody reaction in the tissues, yet this has not produced a hemorrhagic area as in the case of the "Shwartzman phenomenon" when the antigen is introduced by two different routes, (skin and vein). Indurations were also seen when the sera of convalescent typhoid patients were introduced into the skin together with the filtrate. Whether or not the original reaction with the concentrated filtrate is also due to an allergic state is difficult to say but it is a known fact that gram negative bacilli of this group are constant inhabitants of the gastrointestinal canal and this may explain the appearance of the reaction.

We have succeeded in producing the "Shwartzman phenomenon" with our concentrated filtrates, thus confirming these observations with a highly purified product. The reactions were further obtained in animals whose skin was prepared by Shwartzman's filtrate and by our own filtrate and vice versa, indicating that we must be dealing with the same reacting products. The Shwartzman reaction also



occurred in animals in which the skin preparation was made by the intracutaneous injection of filtrate and antiserum, that is, at the site of an inflammatory process on the skin, a hemorrhagic area would appear if followed by an intravenous injection of the filtrate alone.

A single injection of the filtrate and its homologous antiserum never produced a hemorrhagic area. The reason that an intravenous injection of the filtrate is necessary for the production of the reaction is difficult to explain. Subcutaneous injection of the filtrate failed to produce the phenomenon. Filtrates of cultures of organisms of the same group will produce the reaction; whereas filtrates from non-related organisms (enterococci) failed to react in animals whose skins were prepared by filtrates of all the members of the colon-typhoid group.

The products used by us were relatively thermostabile but did not withstand autoclaving for 15 minutes at 15 lbs. pressure, neither did they withstand 30 minutes exposure to ultraviolet light.

Ecker and Rimington have reported on the chemical nature of these products and similar findings were also obtained by Branham (16) and others.

#### SUMMARY AND CONCLUSIONS

The observations of Ecker and Rimington on the production of skin reactions with synthetic medium culture filtrates (concentrated and dialyzed) of organisms belonging to the colon-typhoid group have been confirmed and extended.

The filtrates show a marked degree of thermostability and marked resistance to ultraviolet radiation.

Intravenous injections of the filtrates stimulate the production of precipitins. No precipitins are produced in the sera of rabbits that receive intracutaneous injections only.

At no time during the course of immunization was the skin reaction obliterated. An indurative process was noted at the site of injection (skin) during the course of immunization. Mixtures of the filtrates with their homologous antisera, obtained from animals immunized by the intravenous route, and injected into skin, also failed to obliterate the erythema and edema.

The observations of Shwartzman have been confirmed; the Shwartz-

man phenomenon appears to be group or species specific. Filtrates from enterococci failed to produce the phenomenon. Filtrates of other unrelated organisms have not been studied.

The products of Shwartzman and of Ecker and Rimington appear to be the same.

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# CHANGES IN HUMORAL IMMUNITY OCCURRING DURING THE EARLY STAGES OF EXPERIMENTAL PNEUMOCOCCUS INFECTION\*

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While much has been learned concerning the appearance of immune substances in the blood at the time of recovery from lobar pneumonia and experimental pneumococcus infection, relatively little is known about changes in humoral immunity occurring during the early phases of the disease. That such changes occur and have an important bearing on the inception and evolution of the infection seems highly probable in the light of recent experimental evidence as to the nature of natural resistance to pneumococcus infection (1).

The findings of earlier workers in this field are inconclusive. This is to be explained presumably by the use of methods lacking sufficient delicacy for the detection of normal anti-pneumococcus substances in the blood stream. Wolf (2), Rosenow (3), Tunncliffe (4), and others, testing phagocytic activity by the determination of the opsonic index (Wright), agreed for the most part that the opsonic index was low in severe pneumonia cases at the height of the disease and was followed by a slight rise at the time of crisis. In fatal cases there was a fall in the opsonic index before death. However, these workers used avirulent strains of pneumococci in their tests; whereas the organisms isolated from pneumonia patients were almost invariably virulent and found to be resistant to the opsonic action of the serum. Other workers (5), (6), (7), studying the humoral changes in lobar pneumonia, were unable to demonstrate opsonins, agglutinins, or mouse protective substances, in the blood stream until about the time of crisis. Bull (8), on the other hand, studying the natural resistance of the dog to pneumococcus infection, was able to demonstrate clearing power of normal dogs blood for highly virulent pneumococci. He injected dogs with virulent pneumococcus cultures intravenously and by plating samples of blood at short intervals observed a rapid decrease in the number of pneumococci in the blood stream following injection.

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In fatal cases this diminution was only temporary and was followed by an increase and death from septicemia. He inferred that the clearing of the blood was due to agglutination and the phagocytic action of the circulating leucocytes and the cells of the reticulo-endothelial system, although neither opsonins nor agglutinins could be demonstrated in the dog's serum until the sixth or seventh day of the disease.

Tillett (9), working with Type III pneumococcus, using a similar technique, injected rabbits intravenously and showed that avirulent or R forms were rapidly taken out of the blood stream. With strains of slight virulence, there was a decrease and a subsequent rise in the number of circulating pneumococci. When highly virulent strains were used, there was no diminution but a steady increase in the bacteremia until death of the animal. With the usual methods employed for the demonstration of opsonins and agglutinins, Tillett failed to find evidence of humoral immune properties sufficient to account for the rabbit's ability to destroy the avirulent pneumococci.

Recently Robertson and Sia (10) (11), studying the natural resistance of animals to pneumococcus infection, found by an especially devised technique that the serum and leucocytes of pneumococcus resistant animals such as the dog, cat, pig, etc., possessed marked pneumococcidal properties for highly virulent strains of pneumococci while the blood of pneumococcus susceptible animals as the rabbit and guinea pig was lacking in this action. This property of the serum and leucocytes of resistant animals was found to depend on the presence of opsonins and agglutinins which could be demonstrated by employing a relatively large ratio of serum to microorganisms (1).

The technique of Robertson and Sia, by which it is possible to demonstrate constantly natural humoral immune bodies in the blood of certain animal species, provides a means of studying changes in the circulating defense mechanism during the inception and evolution of pneumococcus infection. The object of the present study has been primarily to follow the early changes in the humoral immune substances of the dog and cat during experimental pneumococcus infection.

### *Methods*

*Infection:* Normal dogs and cats were infected by intrapleural or intraperitoneal injections in the first experiments and by intrabronchial insufflation in the later ones. A virulent Type I strain and a virulent Type II strain of pneumococcus originally isolated from pneumonia cases were used. The virulence was maintained by frequent passage through rabbits.

*Serum:* A blood specimen was drawn before the animal was infected and daily specimens were taken until death or recovery. The blood was allowed to cool in

the ice box and the serum was withdrawn later. Blood cultures and plate counts were done on most of the animals. All sera that contained pneumococci were filtered through an especially designed filter.<sup>1</sup> The sera were preserved in the ice box in bottles containing carbon dioxide.<sup>2</sup> All sera from one animal were tested at one time not later than seven days after the first blood specimen was drawn.

*Opsonic Test:* The method described by Robertson and Sia (1) was followed closely. Homologous leucocytes were obtained from aleuronat exudates withdrawn from the pleural cavity 15 to 18 hours after injection of the aleuronat. Type I pneumococcus was used in the lag phase of growth and Type II pneumococcus in the active growth phase. The pneumococci were sensitized for periods of  $\frac{1}{2}$  hour for actively growing cultures and one hour for cultures in the lag phase in the serum to be tested. A ratio of 50 parts of serum to one part of pneumococcus suspension was employed. The organisms were then sedimented by centrifugation at high speed for  $\frac{1}{2}$  hour; the serum was removed and the pneumococci were taken up in sufficient Locke's solution to make a suspension somewhat more concentrated than that originally added. Wright's capillary pipette mixtures were made using equal parts of sensitized pneumococcus suspension, 1 to 5 normal serum, and a standard leucocyte suspension. The pipettes were incubated 45 minutes and smears were made which were stained with Cross' stain. 100 leucocytes were counted and the percent showing phagocytosis noted.

*Pneumococcal Test:* The pneumococcal promoting substances of the serum were tested by the method described by Robertson and Sia (10). 0.1 cc. dilutions of an actively growing pneumococcus suspension were added to small tubes containing 0.3 cc. serum and 0.1 cc. of a standard leucocyte suspension. The tubes were sealed with corks dipped in paraffin and rotated on an agitator for 15 to 18 hours. Readings were made by determining the amount of methemoglobin formed at the end of 18, 42, and 72 hours. Smears were then made to determine the survival of pneumococci.

## EXPERIMENTAL

### *Generalized Pneumococcus Infection*

*Experiment I:* Cat No. 1, weighing 4000 grams, was bled 10 cc. from the heart and four hours later 0.5 cc. Type II pneumococcus culture was injected intrapleurally. 18 hours subsequently the cat's temperature was 41°C. and it appeared ill. Death occurred on the fourth day. Autopsy revealed peritonitis and a massive empyema of both pleural cavities. The lungs were not consolidated. Culture of the heart's blood was positive for pneumococci. The collected sera were filtered to eliminate any pneumococci present and the opsonic activity of the serum was determined.

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<sup>1</sup> Small pressure filters were made, using the Seitz principle.

<sup>2</sup> According to McAlpine and Valley (12) alexin or complement can be preserved for a long period of time in an atmosphere of CO<sub>2</sub>.

The results are shown in Figure I. The serum of the cat drawn before infection showed marked opsonic activity; 67 per cent of the leucocytes counted showed phagocytosis. 18 hours after infection

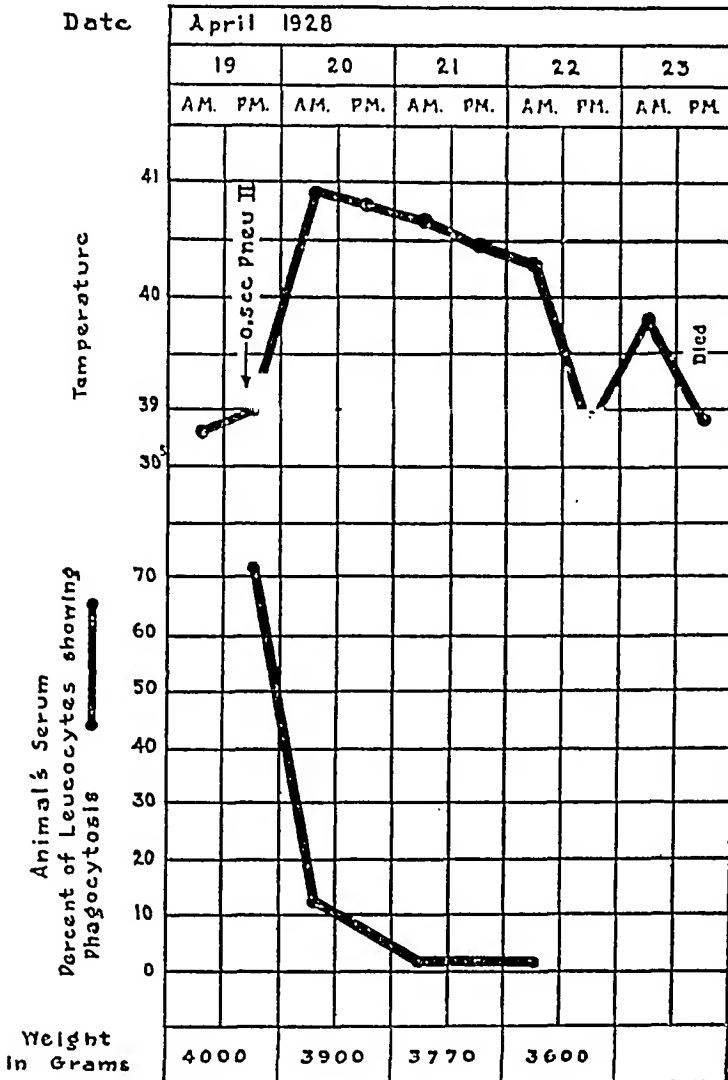


FIGURE I. Cat No. 1. Experimental pneumococcus infection following the intrapleural injection of 0.5 cc. Type II pneumococcus culture.

the opsonic titer had diminished markedly; only 12 per cent of the cells showed phagocytosis. After 48 hours this serum property had disappeared.

*Experiment II:* Dog No. 1, weighing 20.9 kg., was bled 15 cc. from the heart and 4 cc. of an actively growing Type I pneumococcus culture were injected into the right pleural cavity. The next morning the dog appeared sick and the temperature had increased to 41°C. Blood cultures at 18 hours and 42 hours after

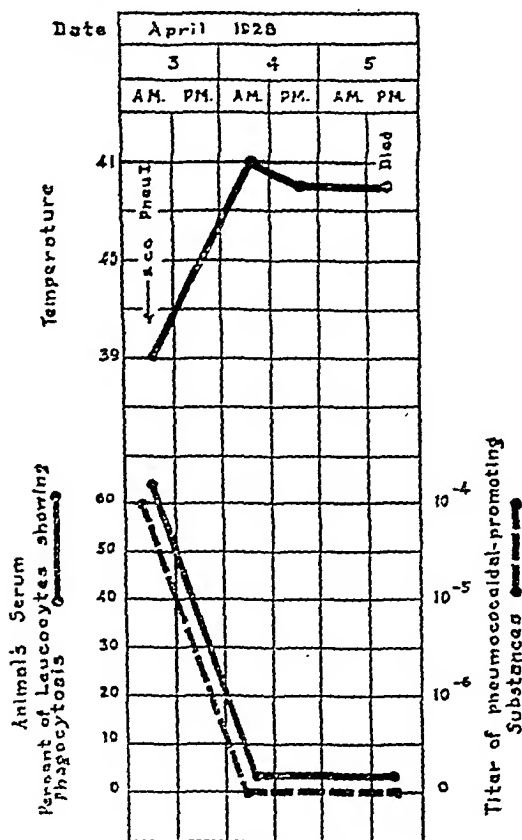


FIGURE II. Dog No. 1. Experimental pneumococcus infection following the intrapleural injection of 2 cc. Type I pneumococcus culture.

infection were positive for pneumococci. The dog died on the third day. Autopsy revealed a massive empyema of both pleural cavities. The lungs were not consolidated.

Before infection the serum contained a high concentration of opsonins. Phagocytosis was noted in 65 per cent of the leucocytes



counted with 60 per cent showing five pairs or more of pneumococci. 18 hours after the inception of infection the opsonic content of the serum had dropped to a point where only 5 per cent of the leucocytes showed phagocytosis with no cells containing more than one or two pairs of pneumococci (Figure II). The pneumococcal promoting action of the serum showed a similar fall in activity (Table I). The serum (0.3 cc.) taken before infection was capable of causing destruc-

TABLE I

*Effect of Pneumococcus Infection on the Pneumococcal-Promoting Activity of the Serum*

Dog serum 0.3 cc. + normal dog leucocytes 0.1 cc. + pneumococcus suspension 0.1 cc.

Kind of serum	Amount of standard suspension		Growth as shown by color changes			Survival of pneumococci stained film
			17 hrs.	42 hrs.	72 hrs.	
Serum before infection	cc.					
	10 <sup>-2</sup>	(0.01)	+++	++++	++++	+
	10 <sup>-3</sup>	(0.001)	++	+++	++++	+
	10 <sup>-4</sup>	(0.0001)	0	0	0	0
	10 <sup>-5</sup>	(0.00001)	0	0	0	0
	10 <sup>-6</sup>	(0.000001)	0	0	0	0
Serum 18 hrs. after	10 <sup>-4</sup>	(0.0001)	++++			+
	10 <sup>-5</sup>	(0.00001)	++++			+
	10 <sup>-6</sup>	(0.000001)	++++			+
	10 <sup>-7</sup>	(0.0000001)	++++			+
Serum 42 hrs. after	10 <sup>-5</sup>	(0.00001)	++++			+
	10 <sup>-6</sup>	(0.000001)	++++			+
	10 <sup>-7</sup>	(0.0000001)	++++			+

tion in the serum-leucocyte mixture of approximately 100,000 pneumococci (10<sup>-4</sup> of the standard suspension) but 18 hours after infection it had lost this property.

*Experiment III:* Dog No. 2, weighing 8.2 kg., was bled 15 cc. from the jugular vein and 2 cc. of an actively growing culture of Type I pneumococcus were injected into the trachea by a needle and syringe. The dog was held so that the culture would run into the right lung. 18 hours later the dog's temperature had risen to 41.1°C. and it appeared very toxic. Death occurred on the third day. Autopsy

revealed an empyema of both pleural cavities. The lobes of the right lung were well collapsed and markedly congested. The left lung resembled the right except that it was more air containing. Microscopical sections showed congestion of the alveolar walls but no leucocytic exudate was present.

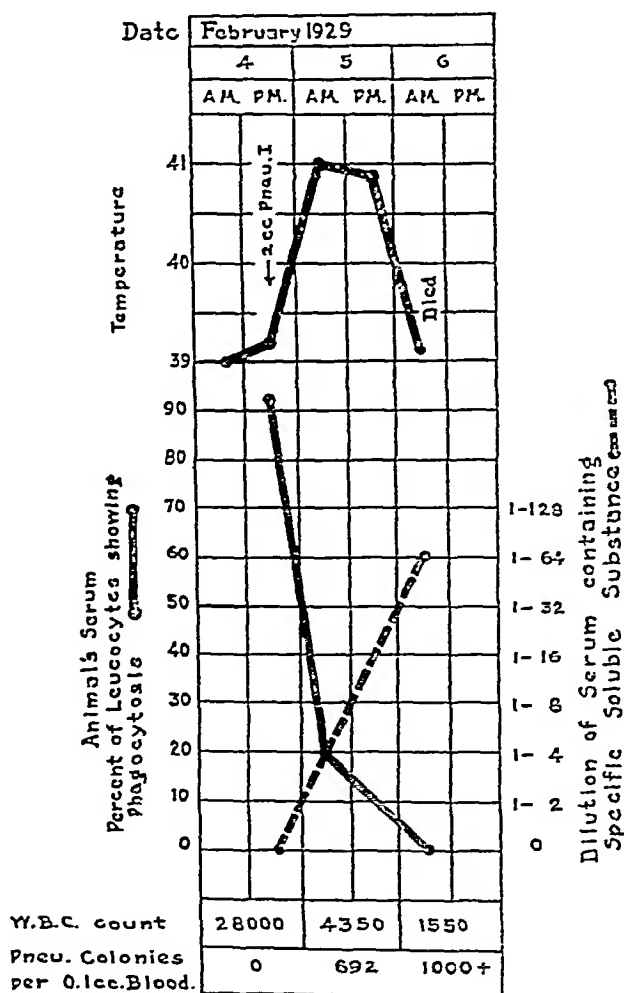


FIGURE III. Dog No. 2. Experimental pneumococcus infection following the intratracheal injection of 2 cc. Type I pneumococcus culture.

The results are shown in Figure III. At the end of 18 hours the opsonins were reduced but there were still enough present to cause a

slight degree of phagocytosis although approximately 6000 pneumococci per cc. were circulating in the blood stream and a demonstrable quantity of specific soluble substance was present in the serum. At the end of 42 hours the opsonins had disappeared completely while the number of pneumococci and amount of soluble substance in the serum had increased. The leucocyte count was reduced from 28,000 before infection to 1,550 on the day of death.

These three experiments, with others that followed a similar course, show that with an overwhelming pneumococcus infection accompanied by early blood invasion there is a rapid decrease in the concentration of humoral immune bodies which disappear entirely by the time of death. In other experiments in which animals recovered after a moderately severe generalized infection this same early diminution of humoral immune substances was observed to occur but the concentration of immune bodies began to rise with the onset of recovery which usually occurred about the fourth day. These immune bodies, appearing in the blood at the time of recovery, are of the so called acquired type as shown by Robertson and his co-workers (13).

In order to determine the nature of this decrease in humoral immune substances in a severe infection, a dog was injected with the filtrate of a 24 hour culture of pneumococcus. According to Avery and Heidelberger (14) the filtrate at this stage contains very little of the nucleoproteins of the pneumococcus but a considerable amount of the specific soluble substance which they have shown to be a carbohydrate, possibly a polysaccharide derived from the capsule of the cell. The experiment follows:

*Experiment IV:* Dog No. 3, weighing 16 kg., was bled 15 cc. from the heart and 50 cc. of filtrate from a 24 hour Type I pneumococcus culture were injected intravenously at 2 P.M. At 3 P.M. a sample of blood was drawn. At 4 P.M. 100 cc. of filtrate were injected intraperitoneally. Blood samples were drawn at 18 and 40 hours after injection.

The results are shown in Table II. There was a decrease in the opsonic action in the sample drawn one hour after injection. After 18 hours there was a slight return toward the initial opsonic titer but it did not return to normal until 40 hours after the filtrate injection. This diminution in opsonic activity is presumably due to the combina-

tion of the specific soluble substance with the natural immune bodies. Sia (15) using normal serum-leucocyte mixtures found that the presence of a very small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixture so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which possess the power to destroy ordinarily such pneumococci in relatively large numbers.

The opsonins in these experiments were highly type specific. The blood of animals infected with Type I pneumococcus did not show a decrease in opsonins when tested with a Type II organism and vice versa. The filtrate injections caused only a decrease in the opsonins for that specific type.

TABLE II

*Effect of Intravenous Injection of Culture Filtrate on Opsonic Properties of the Serum*

Kind of sensitizing serum	Degree of phagocytosis	
	Per cent of leucocytes showing phag.	Per cent showing 5 pairs or more
Serum before injection.....	78	66
One hour after injection.....	37	7
18 hours after injection.....	59	29
48 hours after injection.....	75	52

There has been evidence which has led some investigators (8) to think that a septicemia occurs, not because the natural immune substances have become exhausted, but because the organisms have become adapted to their host and have increased in virulence. In several dogs pneumococci were isolated from the blood stream at different stages of the septicemia and their susceptibility to phagocytosis compared with that of the organism originally used. No differences between them were found.

### *Localized Pneumococcus Infection*

#### *(Lobar Pneumonia)*

A generalized and overwhelming infection as in the experiments above described is not the type of infection found characteristically

in lobar pneumonia. Although there may be a transient bacteremia and in some fatal cases a persistent bacteremia, lobar pneumonia, typically, is a localized pneumonic process. Lamar and Meltzer (16) produced with varying success, a lobar consolidation in dogs by injecting the pneumococcus culture deep into a bronchus by using a tracheal catheter. As a rule the dogs either developed a very light infection or the infection terminated fatally with empyema and a septicemia. With this in mind we attempted to produce a severe infection, yet an infection localized in the lungs. A similar method was employed except that the pneumococci were first suspended in a viscous medium (16 per cent gelatin broth). Because of the high degree of virulence for dogs, from 1 to 3 cc. of culture were sufficient to produce an infection. Five dogs out of nine treated in this manner developed a localized pneumonic infection. The other four died from septicemia and empyema.

*Experiment V:* Dog No. 4, weighing 17.6 kg., was bled 15 cc. from the jugular vein then etherized and a number 8 tracheal catheter was inserted as far as possible into a bronchus. Type I pneumococci from 3 cc. of an 18 hour culture were suspended in 16% gelatin broth solution and injected into a bronchus through the catheter. The next morning the dog appeared ill. The temperature had risen from 38.5°C. to 39.9°C. There was a marked cough, forced expiration, and an increased respiratory rate. X-ray of the chest taken on the third day showed consolidation of the three right lobes and the upper left lobe. The condition of the dog became progressively worse until death occurred on the sixth day. At autopsy the pleural cavities contained no fluid. The right lung was consolidated except for the lower half of the lower lobe which was air-containing. The left upper lobe was congested but air-containing. The left lower lobe appeared normal. The consolidated lobes were comparatively firm and gray in appearance. There was no fibrinous exudate covering the surface. On the cut surface was a grayish thick exudate. Microscopical sections revealed many polymorphonuclear leucocytes within the alveoli. There was only a slight amount of fibrin present. The heart's blood was negative for gram positive diplococci.

The results of this experiment are shown in Figure IV. The most important observation made was the fact the dog died with a negative blood culture. There was an early blood invasion noted 18 hours after infection but the remaining blood cultures proved negative. The pneumococcal promoting power of the blood did not diminish to

an appreciable extent throughout the course of the infection. The opsonic properties of the serum corresponded in general with the pneumococcal titers. Evidently the humoral immune substances in

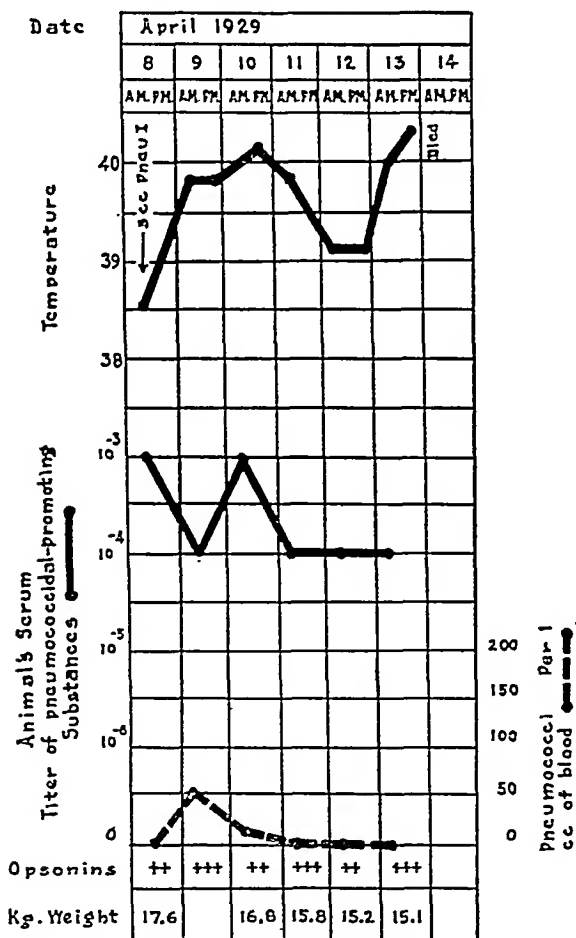


FIGURE IV. Dog No. 4. Experimental pneumococcus infection following the intrabronchial injection of 3 cc. Type I pneumococcus culture.

this case were sufficient to prevent invasion of the blood stream but, on the other hand, did not prevent the spread of the infection within the lung which was lobar in distribution.

*Experiment VI:* Dog No. 5, weighing 16 kg., was bled 15 cc. from the jugular vein, etherized, and pneumococci Type I from 2 cc. of culture were suspended in 16% gelatin broth solution and injected deep into a bronchus by intrabronchial insufflation. The next morning the dog appeared quiet, would not eat, and its

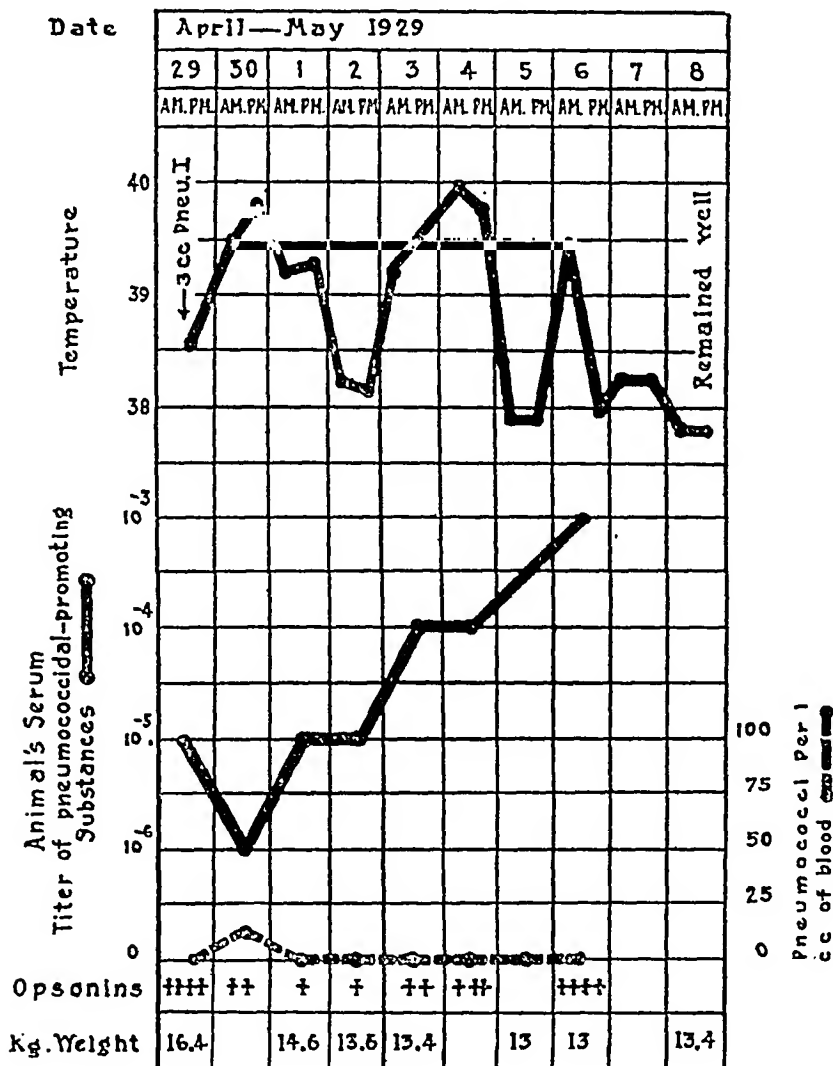


FIGURE V. Dog No. 5. Experimental pneumococcus infection following the intrabronchial injection of 3 cc. Type I pneumococcus culture.

temperature was slightly elevated. X-ray showed marked consolidation of the right lower and middle lobes. The third day the respiration had increased to 92 per minute, there was marked dullness on the right side to percussion, and on auscultation marked bronchial breathing was heard on the same side. On the seventh day the temperature subsided and the condition of the dog improved.

As in the previous case, there was a slight early blood invasion which did not persist after the second day (Figure V). The pneumococcal promoting action of the serum was slightly decreased after 18 hours but there was still an appreciable degree present. On the third day the titer of this serum property had returned to normal and showed a further increase as long as the blood specimens were taken. Tests for opsonic activity showed a more marked decrease in this property but it followed a similar course.

*Experiment VII:* Dog No. 6, weighing 22 kg., was bled 15 cc. from the jugular vein, anesthetized with ether, and 3 cc. of Type I pneumococcus suspension injected deep into a bronchus. The next morning the dog appeared quite sick. The temperature was elevated. X-ray revealed a consolidation of the right lower lobe. The condition remained about the same until the fourth day when signs of fluid developed in the right chest. The dog died on the seventh day. Autopsy revealed a thick grayish fluid in the pleural cavity of each side. The pericardial cavity contained about 75 cc. of like material. Stained smear showed innumerable gram positive diplococci. The lobes of the right lung were well collapsed and dark red in color. The left lower lobe was congested but air-containing. The left upper lobe appeared normal. No true consolidation was seen. Microscopical sections from the lobes of the right lung showed congestion of the alveolar walls and red cells in some of the alveolar spaces. Very few leucocytes were seen.

The humoral immune changes occurring in this dog differed considerably from those in the preceding animals. 18 hours after infection the immune substances had not decreased (Figure VI) although there were 86 pneumococci per cc. in the blood. After 42 hours however, there was a marked reduction in the pneumococcal-promoting substances and a slight diminution in the number of organisms per cc. of blood. By the fourth day of the disease the concentration of immune bodies had shown a rise while the number of circulating pneumococci had fallen to only 16 per cc. of blood. The following day when there were definite signs of fluid in the chest, the titer of the pneumococcal-promoting substances was back to normal but there was a marked increase in the bacteremia; 280 organisms per cc. of blood were noted. The bacteremia increased until death although the pneumococcal-promoting substances were still present.

In attempting to correlate the findings in this animal with the preceding observations, account must be taken of the type of lesion pres-



ent in the lung. Judging X-rays taken 24 hours after the onset of the infection there was a definite beginning localization of the pneu-

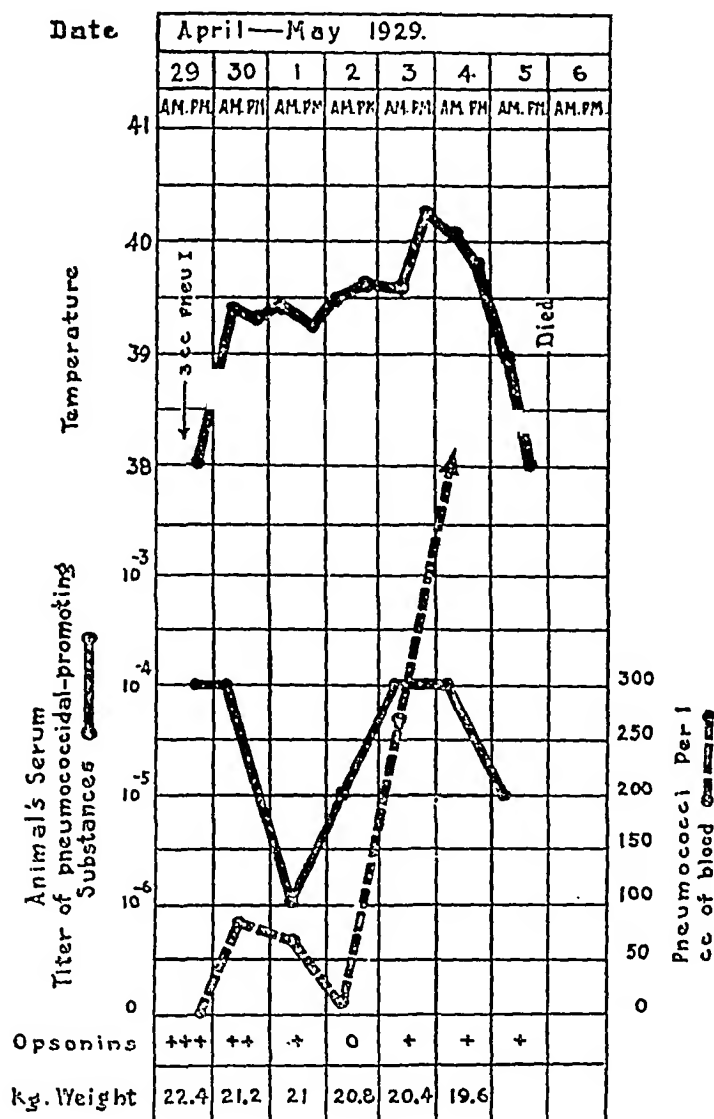


FIGURE VI. Dog No. 6. Experimental pneumococcus infection following intrabronchial injection of 3 cc. Type I pneumococcus culture.

monic process. While blood invasion at this stage was somewhat more marked than in dogs (3) (4), it was by no means as pronounced as in those animals suffering from a generalized pneumococcus infection.

As in the other dogs with lobar pneumonia this initial blood invasion diminished but not as quickly nor did the pneumococci disappear from the blood completely. From this it could be inferred that localization of the lung process did not develop as in the dogs with true lobar pneumonia. The lack of definite pneumonic consolidation found at autopsy bore out this assumption. The terminal state of the blood in which large numbers of pneumococci were circulating in the presence of a considerable concentration of pneumococcal-promoting substances is not susceptible to a satisfactory explanation on the basis of the data available. It would seem most probable, however, that the phagocytic cells of the body were failing to function adequately. While leucocyte counts were not made in this animal, observations on other dogs with overwhelming pneumococcus infection showed a great reduction in the number of circulating leucocytes. The onset of empyema may have produced a depression of functional activity of the phagocytic cells of the body as well. The excess of antibody over antigen might be accounted for by the fact that at this stage of the disease, 4th to 5th day, the immune substances present were of the acquired type and being produced in a concentration much greater than that of the normal antibodies and yet one would expect that if the immune substances were active the pneumococci would be agglutinated and swept out of the blood stream as shown by Bull (8). An alternative possibility could be the presence of some unknown factor operating to prevent the union of antigen with antibody. That the invading pneumococcus had become resistant to the dog's immune bodies seems unlikely in view of studies on this point in other animals.

#### SUMMARY

A study was made of the changes in humoral immunity occurring during the early phases of experimental pneumococcus infection in the dog and cat. The methods devised by Robertson and Sia were employed to demonstrate the presence of anti-pneumococcus properties in the serum of animals naturally resistant to this micro-organism. It was found that with a generalized and overwhelming infection accompanied by early blood invasion, there was a prompt and rapid decrease in the concentration of natural humoral immune bodies which frequently disappeared entirely by the time of death. This same

early diminution of humoral immune substances, opsonins, agglutinins, and pneumococcal-promoting bodies was observed in animals that survived a moderately severe generalized infection but the concentration of immune bodies rose again with the onset of recovery. The decrease in concentration of humoral immune substances during a severe generalized infection appeared to be due to the combination of "S" substance with the normal immune bodies.

When the pneumococcus infection was more localized as in the case of true lobar pneumonia a quite different sequence of events was observed to occur. Several animals, in which extensive lobar pneumonia was produced, showed the presence in quantity of humoral immune bodies in the blood throughout the course of an infection terminating fatally.

These findings suggest that after the inception of pneumococcus infection in the dog and cat the chief function of natural anti-pneumococcus substances in the blood is to limit or prevent blood invasion. When pneumococcal infection is localized these circulating antibodies appear to have little effect either in preventing the spread of the process or determining the outcome of the disease.

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# REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

## I. THE ANTIBODY RESPONSE

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During the course of studies on the effectiveness of *Pneumococcus* antigen under different conditions, unexpected results were observed following the introduction of the bacteria into the skin. It was found that the antibody response under this condition is strikingly different from that which is evoked by intravenous injection. The response of the animal to the intracutaneous injection of pneumococci has been subjected, therefore, to a thorough study. Some of the results have been stated in a preliminary communication (1). Further work is reported here.

### *Methods*

1. *Preparation of Vaccines.* Eight to ten hour broth cultures of *Pneumococcus* were centrifuged at a high rate of speed, the supernatant fluid was discarded, and the organisms were suspended in an amount of saline solution equal to one-tenth of the original volume of culture. Occasionally, the sedimented bacteria were washed 1 to 3 times before final resuspension. The concentrated emulsion of bacteria was then heated for one hour at 56-60°C. and sterility was determined by cultures and intraperitoneal injection of white mice.

2. *Preparation of the Skin.* In the earlier experiments the fur was removed by clipping and shaving, but later this was accomplished by gentle rubbing with a saturated solution of barium sulfite. The latter method proved more satisfactory, since, with care, it left a perfectly clear skin which remained free of fur for a number of weeks. The depilation was carried out on both sides of the body, and, since consecutive injections were made, it frequently became necessary to repeat the removal of fur.

3. *Method of Injection.* The suspensions of heat-killed pneumococci were injected intracutaneously once a week in quantities of 0.2 cc. This amount was equivalent to 2.0 cc. of the original culture. The injections were given alternately on the two sides of the body, each injection being made in a different area of the skin. The

number of injections in the different animals varied. In the majority of instances, the total amount of suspension given was equivalent to 20 cc., or more, of culture. The results obtained, therefore, could be roughly compared with those observed following intravenous immunization of rabbits with similar amounts of culture.

4. *Testing for Antibodies.* Some of the animals were tested for antibody production only after a complete series of ten injections and others at different intervals during the process. Usually the rabbits were bled once a week, immediately preceding each injection. Final specimens of blood were procured 10 days following the last injection. The sera were tested for the presence of type-specific antibodies (anti-S), which agglutinate the encapsulated, or S, cells and precipitate the soluble specific substance derived from them, and also for species-specific antibodies (anti-P or anti-R) which agglutinate only the capsule-free, or R, cells and precipitate the protein derived from any type of *Pneumococcus*.

#### EXPERIMENTAL

Rabbits were injected into the skin with suspensions of heat-killed *Pneumococcus* Type I or Type III, or an R strain derived from Type II *Pneumococcus*. In addition, intracutaneous injections were made with solutions of derivatives of *Pneumococcus*, one of which consisted essentially of a solution of "nucleoprotein" (2) and the other of the supernatant fluid after acid precipitation of the "nucleoprotein" from a solution of pneumococci resulting from repeated freezing and thawing. The strain of Type I *Pneumococcus* employed was virulent for white mice and rabbits, and killed animals of both species when  $10^{-7}$  cc. of culture was administered. The Type III culture killed mice in a quantity of  $10^{-7}$  cc. of culture, but did not kill rabbits in amounts as high as several cc., although in these animals it was capable of producing a transient septicemia. In one experiment a rabbit-virulent strain of Type III *Pneumococcus* was used as antigen. The lethal dose of this strain varied from  $10^{-3}$  to  $10^{-4}$  cc. of culture.

#### *Antibody Response to Type I (S) Pneumococcus*

Several groups of rabbits (sixty animals in all) were given repeated intracutaneous injections of suspensions *Pneumococcus* Type I. The development of antibodies was essentially the same in all the animals so that for the sake of brevity and clarity, one group will be described in detail as typical. The data obtained in the study of this group are represented graphically in Figure 1. The curves for the development of type-specific and species-specific agglutinins were plotted from the averages of the titres of the sera of a group of four rabbits. These rabbits had received 12 intracutaneous injections of 0.2 cc. each of a suspension of heat-killed pneumococci of Type I (S form), a total number of bacteria equivalent to those contained in 24 cc. of culture,

which is more than the amount required for the usual intravenous immunization. The astonishing result was the absence of type-specific agglutinins and type-specific precipitins in the sera of all the animals. In other words, the sera did not cause the agglutination of the bacteria employed in the immunization, nor precipitate the soluble specific substance derived from them. Moreover, this result was obtained not only in this particular group of animals, but in a total of 60 rabbits receiving repeated intracutaneous inoculations of Type I *Pneumococcus* only 7, or about 12 per cent, showed any evidence of type-specific response, and in these instances specific agglutinins were demonstrable only in low dilutions of the serum. In two

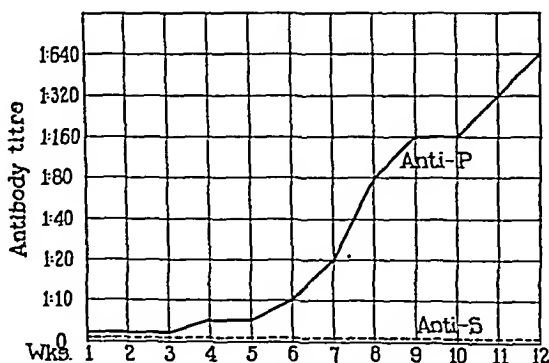


FIGURE 1. The development of antibodies in rabbits immunized intracutaneously to *Pneumococcus*, Type I.

instances agglutination occurred in a dilution of 1:1, in one instance 1:3, in three instances 1:5, and in one case 1:20. Obviously, these results are in marked contrast to those obtained with the sera of animals which have received similar quantities of organisms administered intravenously, in which case agglutination usually occurs in dilutions of 1:80 to 1:160. The lack of type-specific antibodies can be interpreted as evidence of the disintegration of the type-specific antigen after the introduction of the intact cell into the skin.

Despite the fact that type-specific (anti-S) antibodies were absent, the species-specific antibodies (anti-P or anti-R) were found to be present in high degree in the sera of all the rabbits. As illustrated in

Figure 1 the species-specific antibodies, as measured by the agglutination of R cells, are definitely detectable after the third or fourth injection, and after about the sixth injection there is a constant and rapid rise in titre which may reach a serum dilution of 1:640. In the sera of some rabbits, not included in the accompanying curves, the concentration of species-specific antibodies was even higher.

*Antibody Response to Type III (S) Pneumococcus*

In terms of its capacity to stimulate the formation of type-specific antibodies, Type III Pneumococcus is at best a poor antigen. The recent work of Tillett (4) has established the fact that Type III strains

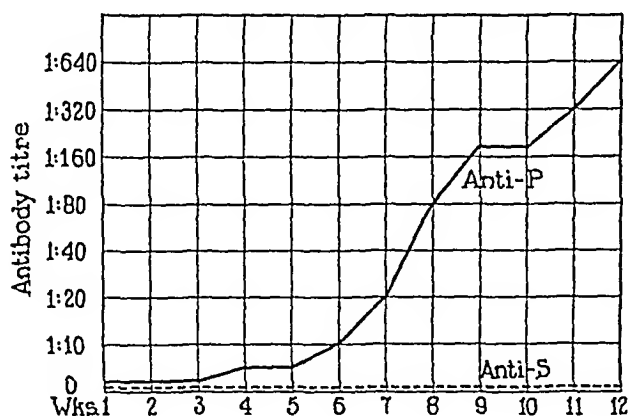


FIGURE 2. The development of antibodies in rabbits immunized intracutaneously to Pneumococcus, Type III.

of marked virulence for mice are usually not only avirulent for rabbits, but in only a small percentage of rabbits (15 per cent) do they elicit a type-specific antigenic response following intravenous inoculation. Moreover, his results indicate that with Type III Pneumococcus there is a parallelism between type-specific antigenicity and virulence. It was to be expected, then, that rabbits immunized intracutaneously with Type III pneumococci would respond very poorly in the production of type-specific antibodies.

In Figure 2, curves are given which illustrate the development of anti-S and anti-R agglutinins. The rabbits were given 12 injections of 0.2 cc. each of a suspension of Type III pneumococci, thus receiving

in all the equivalent of 24 cc. of culture. It will be seen that at no time were type-specific antibodies demonstrable either as agglutinins or precipitins. None of a total of 45 rabbits, studied at different times, possessed anti-S antibodies in their sera during or following repeated intracutaneous injections of Type III pneumococci.

On the other hand, in the sera of all the rabbits species-specific antibodies were demonstrable. As Figure 2 illustrates, the development of anti-R agglutinins in these rabbits closely parallels that observed in the group immunized intracutaneously to suspensions of Type I pneumococci.

#### *Antibody Response to R Pneumococcus*

That R organisms fail to incite the formation of type-specific antibodies has been the common experience of all workers. The intravenous administration of R cells leads only to the development of species-specific antibodies. In studying the antibody response in rabbits following intracutaneous injections, an R strain derived from Type II was employed as antigen, and in each instance the total amount of heat-killed suspension employed was equivalent to at least 20 cc. of culture. None of the rabbits immunized in this manner formed type-specific antibodies. The serum of all the rabbits, however, contained species-specific agglutinins and precipitins in titres comparable to those observed in rabbits immunized to *Pneumococcus* Type I and Type III.

The injection into the skin of the *Pneumococcus* cell, whether R or S, elicits an antibody response which is essentially or entirely of the species-specific variety. It seems likely, therefore, that the type specificity of the S form is destroyed when it is introduced into the skin.

#### *Antibody Response to Soluble Derivatives of Pneumococcus*

In the preceding experiments rabbits were immunized by the inoculation of the intact, formed cells. The studies of Reimann (5) have revealed that the antigenic response of animals to injection of the "nucleoprotein" of *Pneumococcus* is identical with the response to the intact R cells. It was interesting, therefore, to compare the antigenic response of rabbits to the intracutaneous injections of soluble derivatives of *Pneumococcus* with the response to the similar injection



of the intact cells. The soluble derivatives were obtained from an S strain of Type II Pneumococcus. The cells were frozen and thawed repeatedly, and from the resulting solution of bacterial substance, by precipitation with acetic acid in the cold, a material designated for convenience as "nucleoprotein" (2) was obtained. The residual supernatant fluid, after precipitation with acetic acid, was also studied. This supernatant fluid contains, in addition to other substances, the purpura producing material of Pneumococcus (3, 6). The two soluble derivatives were inoculated consecutively once a week into the skin of rabbits in a volume of 0.2 cc. each. The substances were standardized on the basis of protein nitrogen, and rabbits received in toto at least 70 mgm. of the first substance and at least 30 mgm. of the latter considered as protein.

As was to be expected, none of the rabbits injected with either of these two soluble derivatives showed anti-S in their sera. This is in agreement with previous results (7) from this laboratory. On the other hand, all of the rabbits possessed precipitins for the "nucleoprotein" and agglutinins for the R cells but in somewhat lower titre than that demonstrated in the sera of rabbits immunized by either the intact R or S forms of Pneumococcus.

#### *Antibody Response to Intravenous Injections after Previous Intracutaneous Immunization*

The marked differences in antibody responses to type-specific antigens, depending upon whether the intravenous or intracutaneous route of administration was employed, raised the question whether the property of inducing disintegration or dissociation of the type-specific antigen is possessed only by certain tissues, such as the skin, or whether all tissues of the animal body are potentially able to induce this disintegration, and whether, as a result of repeated intracutaneous injections, this property might be so exalted that subsequent intravenous introduction of the bacteria would fail to elicit a type-specific antibody response. In order to determine the answer to this question the following experiment was made. Twelve rabbits were selected which, following repeated intracutaneous immunization, did not show type-specific antibodies in their sera. Six had been immunized with a suspension of heat-killed Type I pneumococci, and six with a suspension

of Type III pneumococci. All twelve were then immunized by intravenous injections of a suspension of heat-killed Type I pneumococci. Following the intravenous immunization, the sera of all 12 rabbits were found to agglutinate Type I cells specifically. The titre in each case reached as high as 1:160. This titre is slightly higher than that usually found in normal rabbits after intravenous immunization alone.

#### DISCUSSION

The significant result of the present study is that the type-specific antigen of pneumococcus does not stimulate the production of antibodies when it is injected into the skin. This is in marked contrast with the results obtained when the injections are made intravenously. Following intracutaneous injections of the whole organisms the resulting antibodies are of the species-specific type. While in the skin the type-specific substances of the bacterial cell, therefore, become ineffective, the remaining constituents of the pneumococcal cell retain the power of stimulating antibody formation. The antibody response to intracutaneous inoculations is the same whether the type-specific cells (S forms), the cells which do not possess type-specific characters (R forms), or the soluble protein constituents of the cell are injected.

Why the type specific antigen of the cell, when injected into the skin, becomes ineffective in stimulating antibody response is not entirely clear. It is possible that in the skin the bacteria are mechanically localized and that, as a result, the cells undergo a disintegration, comparable to autolysis, during which the type-specific antigen is destroyed. It is more likely that the skin possesses a peculiar property of causing disintegration or dissociation of the compound type-specific antigen, so that the latter is no longer able to stimulate the production of antibodies.

Similar failure of pneumococci to stimulate the production of type-specific antibodies when the bacteria are injected into the skin has been noted by others. Goodner (8) found that when live virulent cultures of Type I pneumococci were injected into the skin of rabbits half of the animals failed to develop type-specific antibodies in their sera. Gross (9) has also found that following the intracutaneous injections of small amounts of live pneumococci in rabbits, no agglutinins appeared in the sera. Bull and McKee (10) found that type-specific antibodies

frequently failed to appear in the sera of animals after pneumococci had been instilled into the nose. It is of much significance that in the experiments of these three sets of observers antibodies failed to develop following the injection of live virulent pneumococci, in two cases into the skin, and in the other case into the nose.

#### SUMMARY AND CONCLUSIONS

1. Sixty rabbits were immunized by the repeated injections into the skin of small doses of suspensions of heat-killed Type I pneumococci. In 53 of the rabbits no type-specific antibodies appeared in the serum, and in the remaining seven the titre of these antibodies in the serum was very low. In all cases, however, the sera possessed a high titre of species-specific antibodies.

2. Forty-five rabbits similarly immunized by injections of heat-killed Type III pneumococci also failed to form type-specific antibodies but did form species-specific antibodies.

3. Suspensions of heat-killed R pneumococci and solutions of bacterial substances when injected into the skin stimulated the production of species-specific antibodies, although they failed to stimulate the production of any type-specific antibodies.

4. Animals which had been immunized by intracutaneous injections still possessed the ability to form type-specific antibodies when they were subsequently given intravenous inoculations of type-specific pneumococci.

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# REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

## II. RESISTANCE TO INFECTION

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The results reported in the preceding communication (1) disclose the fact that the antibody responses to the injection of heat-killed pneumococci differ qualitatively, and that the nature of the response is related to the route of administration of the organisms. Pneumococci of Type I, which are invariably effective in stimulating the formation of type-specific antibodies when they are introduced intravenously, were ineffective in producing this type of antibody response in 88 per cent of the rabbits when the injections were made intracutaneously. Under the latter conditions, the antibody response was solely or predominantly species-specific and was no different from the response of rabbits to the intracutaneous injection of S cells of different types, or, indeed, of degraded, non-type-specific R organisms.

The question naturally suggests itself whether rabbits which have received repeated intracutaneous injections of heat-killed pneumococci, and therefore have in their sera no type-specific antibodies, are, nevertheless, actively resistant to infection, and if so, whether this form of acquired resistance is likewise effective against infection by heterologous types of *Pneumococcus*. The question also arises whether the sera of animals immunized by intracutaneous injections will confer passive protection upon white mice against infection by homologous types of *Pneumococcus*. A preliminary report (2) has already been made of the experiments here presented which were undertaken to solve these problems.

### EXPERIMENTAL

Rabbits were immunized by repeated intracutaneous injections. The inoculations were made once a week and consisted of 0.2 cc. of a suspension of heat-killed

bacteria, or the equivalent of 2.0 cc. of broth culture. The degree of resistance to infection was determined 10 to 21 days after the last intracutaneous inoculation by injecting intravenously varying quantities of live virulent cultures of Type I or Type III pneumococci. The Type I strain killed normal rabbits in amounts of  $10^{-7}$  cc. of broth culture, while the Type III strain was fatal in an amount of  $10^{-3}$  cc. to  $10^{-4}$  cc. Samples of blood were obtained from the rabbits immediately preceding infection and the sera were used to determine the titre of type-specific agglutinins and also the power to protect white mice against infection by pneumococci of homologous types.

*Resistance to Infection Following Intracutaneous Immunization with Heat-Killed "S" Cells of Pneumococcus*

*I. Immunization with Type I (S) Pneumococcus*

Forty rabbits were immunized intracutaneously to Type I *Pneumococcus* and tested later for their resistance to infection. In the earlier experiments the resistance to infection by the homologous type of *Pneumococcus* was determined, while in the later experiments the degree of active immunity to infection by some type of *Pneumococcus* other than that used for immunization was tested.

(a) *Active Resistance to Infection by Pneumococcus Type I (Homologous)*. Resistance to infection by the homologous type of *Pneumococcus* was first determined in a group of three rabbits (Table I).

Each had received into the skin 12 injections of suspensions of heat-killed pneumococci, the total number of bacteria injected being equivalent to those contained in 27 cc. of broth culture. Twelve days after the last inoculation all the animals were given intravenously 0.1 cc. of living virulent Type I culture, and all survived. Control, normal rabbits died within 48 hours after receiving 0.0,000,001 cc. of the same culture. The serum of one of the 3 immunized rabbits possessed an agglutinin titre for the type-specific organism of 1:1; sera from the other two rabbits had no agglutinating power. A second group of 9 rabbits received 8 injections of heat-killed pneumococci equivalent to those contained in 16 cc. of broth culture. Subsequently they survived the intravenous injection of live cultures in quantities as large as 0.2 cc., whereas normal rabbits succumbed following injections of 0.0,000,001 cc. In only 3 of the rabbits were type-specific agglutinins demonstrable.

These observations demonstrate that intracutaneous immunization with heat-killed Type I pneumococci is followed by resistance to the intravenous injection of virulent cultures of the same type.

(b) *Active Resistance to Infection by Type III Pneumococci (Heterol-*

. *ogous*). The foregoing experiment shows that rabbits may acquire an effective resistance to infection by the same organism used in immunization, even when demonstrable circulating type-specific antibodies are absent. Consequently it was interesting to determine whether this form of resistance is also effective against infection with pneumococci of heterologous types. Accordingly, in all the following experiments resistance was determined to infection by *Pneumococcus* Type III. In Table I are given results of these experiments in 31 rabbits. It is evident that following intracutaneous administration of suspensions of heat-killed Type I pneumococci, rabbits acquire a marked degree of resistance to infection by virulent Type III organisms. In a general way, the resistance increases with the number of injections. However, in the last experiment given in Table I the rabbits received only one injection and yet they were resistant to 100 lethal doses of *Pneumococcus* Type III.

(c) *Passive Immunity*. It is seen, then, that rabbits may be actively immunized by intracutaneous injections, not only against pneumococci of the type used in immunization but also against pneumococci of other types, and this in the absence of circulating type-specific antibodies. It was interesting to determine whether the sera of these rabbits protected white mice against infection. Protection tests were conducted in the usual manner by the simultaneous intraperitoneal injection of 0.2 cc. of serum and varying dilutions of Type I *Pneumococcus* culture. The sera of 53 animals were tested (Tables I and III); the sera of 42 of them showed no measurable protective properties for mice, while the sera of the remaining 11 exhibited protective power against only very small infecting doses of *Pneumococcus* Type I culture. Of the 11 sera which were protective, 6 contained no type-specific agglutinins while 5 showed type-specific agglutinin titres of 1:1–1:20. On the other hand, of the 42 sera with no protective capacity only one showed type-specific agglutinins, and in this case the agglutination titre was 1:3. It is evident, therefore, that in most instances in which the sera possessed protective power this was associated with the presence of type-specific agglutinins. On the other hand, in most of the animals the sera possessed no protective power for mice and no type-specific agglutinins. It is possible, however, as the work of Tillett (3) suggests, that these sera might have protective power for rabbits,

TABLE I

*Resistance of Rabbits to Infection Following Intracutaneous Immunization with Heat-Killed Pneumococcus, Type I*

Number of rabbits	Number of injections	Total amount injected	Immunity to Type I (homologous)			Immunity to Type III (heterologous)			Protective capacity for mice		Anti-S titre
			cc.	Survived	Died	cc.	Survived	Died	Number of sera	Degree of protection	
		cc.									
3	12	27	0.1	3	—	—	—	—	2 1	none $10^{-5}$	negative 1-1:1
9*	8	16	0.2	3	—	1.0	1	1	1 1 2	$10^{-2}$ $10^{-3}$ $10^{-4}$	1:20 1:1 1:5
			0.1	3	—	0.8	2	1	2	$10^{-5}$	negative
									1	$10^{-6}$	negative
			0.01	3	—	0.5	3	—	2	none	negative
5	7	14	—	—	—	—	—	—	5	none	negative
4	14	29	—	—	—	1.0	2	—	4	none	negative
						0.5	2	—			
5	10	20	—	—	—	1.0	—	2	5	none	negative
						0.8	—	2			
						0.5	1	—			
2	5	10	—	—	—	1.0	1	—	2	none	negative
						0.5	1	—			
2	10	20	—	—	—	1.0	1	—	1	$10^{-6}$	negative
						0.5	1	—	1	none	negative
9	1	2	—	—	—	0.5	—	1	9	none	negative
						0.3	—	2			
						0.1	—	2			
						0.01	2	—			
						0.001	2	—			

M. L. D. of Type I culture for normal rabbits,  $10^{-7}$  cc.; for mice  $10^{-7}$  cc.

M. L. D. of Type III culture for normal rabbits,  $10^{-4}$  cc.; for mice  $10^{-7}$  cc.

\* In this instance, all nine rabbits were tested first for resistance to Type I infection; and later to Type III.

but this question has not been studied. He observed that, following intravenous immunization with R pneumococci, rabbits not only acquired an active resistance but that the sera of these animals, passively transferred to other rabbits, protected them against infection with pneumococci of every type. These sera, however, afforded no protection to mice against pneumococcal infection.

## *II. Immunization with Type III (S) Pneumococcus*

Thirty-eight rabbits were immunized by intracutaneous injections of heat-killed Type III pneumococci (Table II). These animals were subsequently tested for active immunity to virulent cultures of Type I or Type III *Pneumococcus*.

(a) *Active Immunity to Infection by Type III (Homologous) Pneumococcus*. Resistance to infection by pneumococci of the homologous type was tested in 9 rabbits.

They were immunized by 8 injections into the skin of heat-killed pneumococci Type III, the total number of bacteria corresponding to those contained in 16 cc. of broth culture. All the animals were subsequently infected by intravenous injections of varying amounts of culture in quantities as large as 1.0 cc. The culture employed killed normal rabbits in doses of 0.0001 cc. One of the animals died and 2 survived after receiving 0.5 cc. of culture, 2 died and 1 survived after 0.8 cc., and 1 died and 2 survived after 1.0 cc. of culture. In the 4 animals which succumbed, death was delayed from the seventh to the tenth day after the infection, and in each instance, the post-mortem examination revealed a massive pericarditis and pleurisy. The delayed death and localization of the infection may be considered to be an expression of active immunity.

It is obvious that these animals showed a considerable degree of active immunity to infection with homologous organisms.

(b) *Active Immunity to Infection by Type I Pneumococcus (Heterologous)*. Twenty-eight rabbits which had been immunized by the intracutaneous injection of heat-killed pneumococci (Type III) were later tested for their immunity against infection with heterologous (Type I) pneumococci (Table II). The culture employed in the tests was of such a virulence that 0.0,000,001 cc. injected intravenously regularly killed normal rabbits. The immunized animals received doses of from 0.001 to 0.2 cc. Of the 28 immunized animals receiving these large doses, 22 recovered and only 6 died. It is evident, therefore, that the animals immunized by intracutaneous injections had



acquired a marked resistance against pneumococci of a heterologous type.

(c) *Passive Immunity.* It is well known that only in rare instances is the serum of a rabbit immunized intravenously to Type III Pneu-

TABLE II

*Resistance of Rabbits to Infection Following Intracutaneous Immunization with Heat-Killed Pneumococcus, Type III*

Number of rabbits	Number of injections	Total amount injected	Immunity to Type III (homologous)			Immunity to Type I (heterologous)			Protective capacity for mice		Anti-S titre
			cc.	Survived	Died	cc.	Survived	Died	Number of sera	Degree of protection	
9	8	16	1.0	2	1						
			0.8	1	2				9	none	negative
			0.5	2	1						
3	12	27	—	—	—	0.01	3	—	3	none	negative
5	7	14	—	—	—	—	—	—	5	none	negative
4	14	29	—	—	—	0.1	1	1			
						0.01	2	—	4	none	negative
5	10	20	—	—	—	0.2	—	1			
						0.1	1	—	5	none	negative
						0.01	1	1			
6	7	14	—	—	—	0.1	1	1			
						0.01	2	—	—	—	—
						0.001	2	—			
5	7	14	—	—	—	0.1	2	—			
						0.01	1	1	5	none	negative
						0.001	1	—			

mococcus protective for white mice against infection with homologous organisms. In the experiments here recorded (Table II) the sera of 37 rabbits which were immunized intracutaneously with suspensions of heat-killed pneumococci Type III were tested for their protective power in white mice, and in no instance did these sera confer any

measurable protection. Moreover, in none of the sera were any type-specific antibodies demonstrable.

According to the recent work of Sia (4) normal pig serum, though lacking type-specific agglutinins, protects mice against infection by *Pneumococcus* of any type. The special technique which he employed was used in retesting six of the sera mentioned above. Three of these sera were from the animals immunized by the injection of Type III pneumococci and three from the rabbits immunized with Type I pneumococci. The serum was injected intraperitoneally into the mice 4 hours preceding the inoculation of the live culture. No protective power could be demonstrated in any of these sera by this method.

#### *The Rate of Development and Duration of Active Immunity Following Intracutaneous Injections*

Having determined that rabbits, following repeated intracutaneous immunization, acquire a marked resistance to infection by *Pneumococcus* of any type, observations were next made to determine how rapidly this form of active immunity develops and how long it persists.

Fourteen rabbits were immunized, employing a varying number of intracutaneous injections and different amounts of heat-killed bacteria (Table III (a)). Each of two animals received from 1 to 7 injections of the bacteria, corresponding to 2 cc. to 14 cc. of Type I pneumococcal culture. Three weeks after the last injection, the immunity to infection was measured against Type III pneumococci.

The results show that even after one intracutaneous injection of pneumococci, in an amount corresponding to 2.0 cc. of culture, rabbits may show a distinct resistance to infection with heterologous pneumococci. Of the 14 rabbits, the sera of only 2 showed any anti-S agglutinating power. In one rabbit, which had received 4 injections, the agglutination titre of the serum was 1:3, and in the other, which had received 7 injections, the titre was only 1:5. The protective action of these sera for mice was very low and irregular, only 3 sera affording minor protection against homologous infection. Curiously enough, the 2 sera possessing specific agglutinins afforded no protection to white mice.

Six rabbits of a second group (Table III (b)) were each immunized

TABLE III

*Rate of Development and Duration of Immunity in Rabbits Immunized with Heat-Killed Pneumococcus Type I by the Intracutaneous Route*

*(a) Rate of Development*

Number of rabbits	Number of injections	Total amount injected	Immunity to Type III (heterologous)			Protective capacity for mice		Anti-S
			cc.	Survived	Died	Number of sera	Degree of protection	
2	1	2	0.01	1	—	2	none	negative
			0.01	1	—			
2	2	4	0.1	1	—	2	none	negative
			0.3	1	—			
2	3	6	0.3	1	—	2	none	negative
			0.5	1	—			
2	4	8	0.5	1	—	1	none	negative
			0.5	1	—	1	none	1:3
2	5	10	0.5	1	—	1	none	negative
			0.8	1	—	1	10 <sup>-5</sup>	negative
2	6	12	0.8	1	—	1	10 <sup>-6</sup>	negative
			1.0	1	—	1	none	negative
2	7	14	0.5	—	1	1	none	negative
			1.0	1	—	1	none	1:5

*(b) Duration of Immunity*

*Animals Immunized with Pneumococcus, Type III*

Number of rabbits	Number of injections	Total amount injected	Immunity to Type I (heterologous) after 6 months			Protective capacity for mice	Anti-S
			cc.	Survived	Died		
1	8	16	10 <sup>-1</sup>	—	1	All sera negative both 10 days and 6 months after final injection	
1	8	16	10 <sup>-2</sup>	—	1		
1	8	16	10 <sup>-3</sup>	—	1		
1	8	16	10 <sup>-4</sup>	1	—		
1	8	16	10 <sup>-5</sup>	1	—		
1	8	16	10 <sup>-6</sup>	1	—		

by intracutaneous injections of a suspension of Type III organisms corresponding to a total amount of 16 cc. of culture. Six months after the final injection the animals were infected with Type I Pneumococcus culture in amounts varying from one-tenth to one-millionth cc. As shown in the table the animals were resistant to infection with one-ten-thousandth cc. It appears, therefore, that resistance to heterologous infection still persists after six months although at this time it is considerably diminished.

*Resistance to Infection Following Intracutaneous Immunization with Suspensions of Heat-Killed R Cells of Pneumococcus*

The R strain used for immunization was originally derived from a Type II Pneumococcus. Rabbits were immunized by intracutaneous injections of suspensions of heat-killed pneumococci in a manner similar to immunization with S cells. The animals received amounts of suspension equivalent to those usually employed in intravenous immunization. Resistance to infection was measured by injecting intravenously different quantities of a virulent culture of Type I pneumococci. The results given in Table IV show that 4 rabbits immunized to R pneumococci survived following the injection of 0.01 cc. or 0.1 cc. of a Type I Pneumococcus culture which killed normal rabbits in a dilution of 0.0,000,001 cc. The sera of the rabbits immunized with R organisms derived from Type II pneumococci afforded white mice no protection against infection by Type II pneumococci. The sera also did not agglutinate Type II pneumococci.

*Resistance to Infection Following Intracutaneous Immunization with Soluble Derivatives of Pneumococcus*

It has previously been shown that following intracutaneous immunization with S or R pneumococci, rabbits acquire an active immunity to infection by homologous and heterologous types of pneumococci. It was also shown in a previous paper (1) that the antibody response in the majority of rabbits is the same (species-specific) whether intracutaneous immunization is brought about by the injection of formed cells or by the injection of soluble derivatives of Pneumococcus. The present observations were made to determine whether, following intracutaneous injections of solutions containing soluble derivatives of

Pneumococcus, rabbits become actively immune to infection with living cultures.

The animals received (a) a solution of "nucleoprotein," and (b) the supernatant fluid after acid precipitation of the "nucleoprotein" from a solution of pneumococci resulting from repeated freezing and thawing. These solutions were prepared from a culture of Type II Pneumococcus in the same manner as described in the previous paper (1).

The rabbits receiving "nucleoprotein" were given repeated injections, the total amount of protein administered to each animal, as estimated by nitrogen deter-

TABLE IV

*Resistance of Rabbits to Infection Following Intracutaneous Immunization with R Forms and with Soluble Protein Derivatives of the Cell*

Antigen administered	Number of rabbits	Quantity of antigen	Immunity to Type I (heterologous)			Protection conferred by sera on mice (homologous infection)		
			cc.	Survived	Died	Number of sera	Degree of protection	Anti-S titre
R Pneumococcus	4	20 cc.	$10^{-1}$	2		4	none	negative
Derived from Type II "S"			$10^{-2}$	2				
Nucleoprotein	4	70 mgm.	$10^{-3}$	—	1	3	none	negative
Derived from Type II "S"			$10^{-4}$	—	1			
			$10^{-5}$	—	1			
			$10^{-6}$	1	—			
Supernatant after removal of nucleoprotein	4	30 mgm.	$10^{-3}$	—	1	3	none	negative
			$10^{-4}$	—	1			
			$10^{-5}$	—	—			
			$10^{-6}$	1	—			

mination, being 70 mg. Three weeks following the last immunizing injection, they were given intravenously varying quantities of a virulent Type I Pneumococcus culture. The results, as shown in Table IV, indicate that the immunity induced in these animals was extremely slight; indeed it may be doubted whether any increased resistance was demonstrated. In the sera of none of these animals were type-specific antibodies demonstrable and only one of the sera possessed any protective power for mice, in this case of minimal degree.

The rabbits receiving the second material were given repeated injections, the total amount of solution containing 30 mgm. of protein as estimated on the basis of nitrogen determinations. Active immunity was determined by observing the

results of the subsequent injection of different amounts of Type I *Pneumococcus* culture. The data are given in Table IV and it is seen that little immunity follows the intracutaneous injection of this material. The sera of none of the rabbits possessed type-specific antibodies and the serum of only one of them protected mice against a minimal infective dose. The sera of these rabbits, however, did contain species-specific antibodies, as was shown by their power to precipitate the "nucleo-protein" and to agglutinate R cells.

The results show, then, that repeated injections of soluble derivatives of *Pneumococcus* into the skin do not render rabbits resistant to infection. Tillett (5) has already pointed out that rabbits do not acquire an active immunity following intravenous immunization with solutions of *Pneumococcus*.

TABLE V

*Protective Property of Sera of Rabbits Immunized Intravenously after Previous Intracutaneous Immunization*

Number of rabbits	Previous immunization	Later immunization	Protection titre for mice	
			Number of sera	Degree of protection
6	Intracutaneous Type I	Intravenous Type I	3	cc. 0.2
			3	0.1
6	Intracutaneous Type III	Intravenous Type I	3	0.2
			2	0.1
			1	0.01

*Protective Properties of Sera of Rabbits Immunized Intravenously Following a Previous Intracutaneous Immunization*

It was shown in the preceding communication (1) that previous intracutaneous immunization does not render rabbits incapable of forming type-specific antibodies when they are subsequently given intravenous injections. It was interesting to determine the protection titre of the sera of rabbits immunized first by intracutaneous inoculations and later by intravenous injections. The data presented in Table V show that 12 rabbits were originally immunized by intracutaneous injections of suspensions of heat-killed pneumococci. Six were immunized to Type I and six to Type III pneumococci. At the end of the immunization, none of the sera conferred protection upon

white mice to infection by pneumococci of the respective types, and none contained type-specific antibodies. After a subsequent immunization by intravenous injections of Type I pneumococci the sera of all 12 rabbits not only showed a high agglutination titre (about 1:160) but all conferred a high grade of passive protection upon mice against infection by *Pneumococcus* Type I.

#### DISCUSSION

The results of the preceding communication (1) disclose the fact that immunization by the intracutaneous injection of suspensions of heat-killed pneumococci, whether S or R, incites the formation of species-specific antibodies in all rabbits. In the animals immunized with Type III pneumococci no type-specific antibodies were found, and in the rabbits immunized with Type I pneumococci antibodies appeared in only 12 per cent of the rabbits studied. The present study reveals that following intracutaneous immunization rabbits acquire a marked degree of resistance to intravenous infection by pneumococci, and that this is true whether the pneumococci injected be of the same type as those employed in immunization or of a different type. This active immunity is effective even in the absence of type-specific agglutinins.

In general, the sera of rabbits immunized by intracutaneous injections fail to protect white mice against infection by *Pneumococcus*. However, when pneumococci of Type I are employed in the immunization, about 20 per cent of the sera studied conferred on white mice some degree of protection against infection.

Tillett was the first to point out (5) that the intravenous immunization with R or S cells of any type of *Pneumococcus* renders rabbits actively immune to infection with Type III *Pneumococcus*. In a subsequent communication Tillett showed (3) that although the sera of rabbits intravenously immunized to R or S pneumococci failed to protect white mice against infection by *Pneumococcus*, nevertheless, these sera did confer protection upon normal rabbits. Later Goodner (6) reported that rabbits recovering from an intracutaneous infection with live virulent cultures of Type I *Pneumococcus* were subsequently immune to infection with pneumococci of the same type. More recently Bull and McKee (7) found that following immunization of

rabbits by subcutaneous and intravenous injections of pneumococci of Type II or Type III and by similar injections of a strain of pneumococci of Group IV the animals were actively immune, and that this immunity was effective against intranasal or intravenous injections of pneumococci of Type I. Species-specific antibodies were present in the sera of all the animals. Type-specific agglutinins were present in the sera of the rabbits immunized against *Pneumococcus* of Type II and in the serum of the animals immunized against a *Pneumococcus* belonging in Group IV. In previous papers (8, 9) Bull and McKee had demonstrated that rabbits in which cultures of Type I pneumococci had been instilled into the nose were subsequently actively immune to infection with the homologous organism, and this in the absence of demonstrable type-specific antibodies.

On the other hand, the intracutaneous injections of soluble derivatives of pneumococci in rabbits is not followed by an active resistance to infection with these organisms and the sera of these animals fail to protect white mice against infection.

The observations made in this study indicate, then, that an active and broad resistance to infection by *Pneumococcus* may be present in the absence of circulating type-specific antibodies. Active immunity to pneumococcal infection is usually considered to be associated with the development of demonstrable antibodies which in one way or another react specifically with the bacteria themselves. The sera of animals immunized in the usual manner by repeated intravenous injections usually afford passive protection against infection by corresponding types of *Pneumococcus* to animals of various species and the passive protection is considered to depend upon the transfer of type-specific antibodies. In rabbits immunized by intracutaneous injections, however, the active resistance to infection which develops has been found to be unrelated to type-specificity as is evidenced both by the absence of type-specific antibodies in the serum and by the fact that the resistance to infection which these animals exhibit is effective, not only against pneumococci of the homologous type, but also against infection with pneumococci of heterologous type. Moreover, the sera of these animals do not, as a rule, confer passive protection upon white mice against infection even by homologous types of pneumococci. It is possible that in the one case the resistance is dependent upon the development of a new property, an acquired *active immunity*, and that



in the other case the resistance depends upon an exaltation of a naturally occurring property, an increase in natural *resistance to infection*, but this cannot be determined from the data at hand.

#### SUMMARY AND CONCLUSIONS

1. Injection of suspensions of heat-killed pneumococci into the skin of rabbits is followed by an active immunity which is effective against intravenous infection by homologous and heterologous types of *Pneumococcus*.

2. This form of active immunity may be induced by the injection of S or R strains of *Pneumococcus*.

3. Intracutaneous immunization with soluble derivatives of *Pneumococcus* does not induce active immunity to infection.

4. The sera of seventy-nine per cent of the rabbits immunized to Type I *Pneumococcus* by intracutaneous injections afforded no protection to mice against infection with pneumococci.

5. None of the sera of rabbits intracutaneously immunized to the type-specific Type III (S) pneumococci, to R cells, or to soluble derivatives of *Pneumococcus* protected white mice against infection.

6. The sera of rabbits immunized first intracutaneously and subsequently intravenously possess a high titre of protective antibodies.

7. It may be concluded that when type-specific pneumococci are injected into the skin they lose the property of stimulating an active immunity of a specific type and of stimulating the production of type-specific antibodies, but they act just as do the degraded or R forms, causing the animals to become resistant to infection with pneumococci of all types without the development of any type-specific antibodies in the serum.

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# REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

## III. REACTIONS AT THE SITE OF INJECTION

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It has been shown in a preceding paper (1) that the repeated intracutaneous injection of heat-killed pneumococci in rabbits stimulates the formation of species-specific, rather than type-specific, antibodies.

When Type I *Pneumococcus* was used as antigen, a small number (12 per cent) of the animals did develop a low serum titre of type-specific antibodies, but in all other instances, type-specific antibodies failed to appear in the serum. In a general way, moreover, the antibody response was the same when either S or R cells, or even soluble derivatives of *Pneumococcus*, were administered intracutaneously. Following the injections of S or R organisms, rabbits acquired a marked degree of resistance (2) which was effective against intravenous infection by any type of *Pneumococcus*. The injection of soluble derivatives, on the other hand, induced the development of no active immunity.

When rabbits received an intracutaneous injection of a suspension of heat-killed pneumococci a reaction always occurred at the site of injection, and as the inoculations were repeated week after week with a fixed quantity of bacteria, the local reactions following the injections manifested a change in size, character, intensity, and duration. The occurrence of reactions was in no way related to the type or variety of *Pneumococcus* employed in the injections nor was the intensity of the reaction dependent upon the culture employed. A study has been made of these local reactions.

### EXPERIMENTAL

Inoculations of 0.2 cc. of suspensions of heat-killed pneumococci<sup>1</sup> were made into the skin over the flanks. Repeated injections were made alternately on the two

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<sup>1</sup> In all cases, unless otherwise stated, the suspensions of heat-killed pneumococci were prepared in such manner that 1 cc. of the suspension contained the bacteria from 10 cc. of an 18 hour broth culture.

sides and a new location was chosen each time. Descriptions and measurements of the local reactions were made each day until a complete and final disappearance had taken place. The size of the area of skin involved in the reaction, as determined by actual measurement, has been held to be the only available method of quantitative estimation of the intensity of a given reaction. Usually the reactions reach their greatest size about 48 hours after the injection. Consequently, the sum of the two largest diameters, as measured at this time, has been considered to be an index of the intensity of any given reaction. This has permitted a comparison of the reactions occurring after the first and those after the subsequent injections.

### *Skin Reactions Following Initial Injection*

The intracutaneous injection into a rabbit of 0.2 cc. of a suspension of heat-killed pneumococci is followed within a few hours by the appearance at the site of injection of a circumscribed, slightly raised and indurated nodule, measuring from 1 cm. to 1.5 cm. in diameter. The skin over this area is reddish in color. The reaction reaches its maximum size, of from 1 cm. to 1.5 cm. in diameter, in about 36 to 48 hours. It gradually becomes smaller and disappears within 4 to 5 days without the skin breaking down or becoming necrotic. There is some variation in the intensity of the reaction in individual animals but usually the course is about as stated above. Occasionally, regression occurs more rapidly, within two or three days, or, more rarely, complete disappearance is delayed, even for as long as two weeks or more.

### *Skin Reactions Following Repeated Injections*

When after an interval of a week, a second injection is made into the skin the reaction which follows differs somewhat in intensity and character from that observed following the first injection. The modification of the reaction becomes more marked as the injections are repeated from week to week. Reactions of the greatest intensity occur usually after the fourth to the sixth injection.

In the more intense reactions the area of the skin involved reaches 4 cm. to 6 cm. in diameter. The skin is markedly elevated and of a deep red or purplish hue, and at times appears mottled with small yellow areas. Surrounding the raised area there is usually an areola of erythema varying from 0.2 cm. to 1.5 cm. in breadth. Outside of this the skin may be slightly edematous over a considerable area,

especially on the ventral margin. Not infrequently necrosis of the skin occurs, with discharge of purulent material. In case necrosis does not occur, the disappearance of the lesion is delayed. The time required for regression is apparently related directly to the intensity of the reaction.

To illustrate the change which gradually occurs in the intensity and character of the reactions following repeated injections, charts have been made which give the size of the area involved in each reaction, the length of time required for its disappearance, etc.

TABLE I

*The Duration of Successive Reactions Following Repeated Intracutaneous Injections of 0.2 Cc. Suspensions of Pneumococcus*

Rabbit No.	Number of the injection									
	1	2	3	4	5	6	7	8	9	10
12-56	9*	15	24	23	33	14	12			
12-57	17	16	24	22	37	15	21			
12-58	16	12	22	16	21	7	6			
12-59	22	20	59	28	44	10	10			
12-60	20	17	50	58	37	16	12			
13-89	7	62	55	50	40	31	21			
13-90	10	22	22	21	17	9	16			
13-69	5	21	25	36	32	22	13	9	5	5
13-70	4	18	34	36	31	12	5			
13-72	5	14	43	35	29					

\* The figures represent the number of days required for the disappearance of the reactions.

After reactions of maximum intensity occur, following the fourth or fifth injection, the reactions to subsequent injections vary considerably as regards size. At first, there occurs a gradual decrease but later marked variations occur. These variations are partly explained by the fact that the reactions to later injections are frequently less well circumscribed, and, therefore, less easily measured than are the reactions following the earlier injections. This variability in size is shown in Figures 1, 2, and 3.

In Table I is recorded the number of days required for complete disappearance of each reaction which follows each of ten consecutive

injections made at intervals of a week. It will be seen that the number of days required for the lesions to disappear becomes progressively greater up to about the fourth injection, when the duration of the lesion again becomes diminished. This is also shown in the curve in Figure 1. Any marked irregularities which occurred were usually due to necrosis of the lesions and discharge of pus, with consequent more rapid resolution. Although reactions to later injections became less intense, of smaller size and of less duration, they never completely fail to appear, even though many injections are made. The above observations indicate that the reactivity of the skin to injections of pneumococci becomes gradually increased at least, up to the time four or five weekly injections have been made.

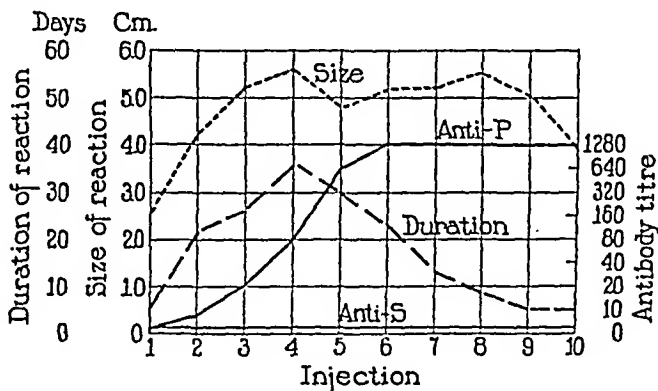


FIGURE 1. The size and duration of the skin reactions following each of a series of injections of heat-killed pneumococci, and the development of antibodies in the serum.

It seemed, however, important to attempt to demonstrate this in another manner, namely, by determining the minimum amount of bacterial suspension required to produce a visible reaction after the animal had received three previous injections, as contrasted with the minimal amount required to produce a reaction in the animal previously untreated.

Each of eight animals received intracutaneous injections of suspensions of heated pneumococci representing amounts of culture varying from 2 cc. to 0.0001 cc. In all cases the volume of fluid injected was 0.2 cc. It was found that reactions occurred when suspensions containing the bacteria from 1 cc. to 0.1 cc. of culture had been injected, but not when suspensions containing smaller numbers

of bacteria were employed. Each of the eight rabbits now received at the end of each of the two subsequent weeks the usual injections of 0.2 cc. of suspensions and the usual reactions were observed. At the end of the following week each of the rabbits received injections of varying amounts of suspension corresponding to those employed in the first test. It was now found that two of the animals showed reactions where amounts of suspension containing the bacteria from .001 cc. of culture were injected, one showed a reaction with an amount as small as that representing .01 cc. of culture, and five showed reactions with amounts of suspensions representing 0.1 cc. of culture. These results are shown in Table II.

After these animals had received injections for three weeks they reacted to amounts of culture smaller than those which were neces-

TABLE II

*Minimal Doses of Suspensions of Pneumococcus Which Elicit Skin Reactions in Normal Animals and in Animals Previously Injected*

Rabbit No.	Minimal dose eliciting reaction at	
	First injection	Fourth injection
	cc.	cc.
8-47	0.01*	0.01
8-48	0.01	0.0001
8-49	0.2	0.01
8-50	0.01	0.0001
8-51	0.1	0.001
8-52	0.01	0.01
8-53	0.1	0.01
8-54	0.1	0.01

\* These quantities of bacterial suspension represent a tenfold concentration of culture.

sary to elicit a reaction originally. The previous observations of an increase of reactivity following successive injections were therefore confirmed by these tests.

#### *Relation of the Increasing Skin Reactivity to the Development of Circulating Antibodies*

An attempt was made to correlate if possible the increasing skin reactivity described above with the appearance of circulating antibodies. As has already been pointed out in previous communications (1, 2) the appearance of type-specific and protective antibodies following intracutaneous injections of suspensions of heat-killed pneu-

mococci occurs only occasionally and then only when pneumococci of Type I have been employed in the injections. On the other hand, the present study shows that all rabbits develop an exalted skin reactivity following successive intracutaneous injections of heat-killed pneumococci, no matter what type of *Pneumococcus* is employed. Consequently, skin reactivity cannot be related directly to the appearance of type-specific antibodies in the blood serum.

On the other hand, as has previously been shown, following repeated intracutaneous injections of heat-killed pneumococci all rabbits develop in their serum a high titre of species-specific antibodies. A study of increased skin reactivity and the presence of species-specific anti-

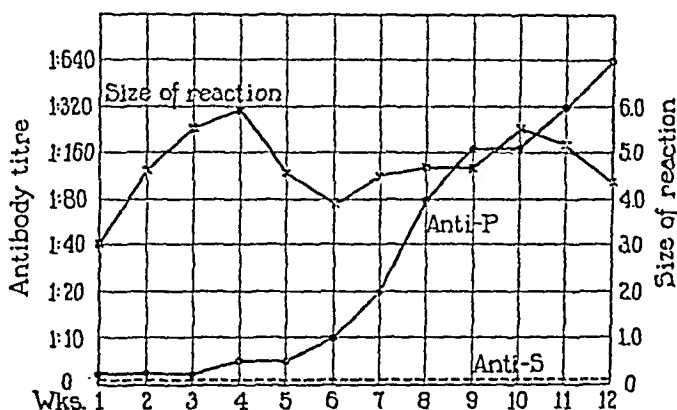


FIGURE 2. The development of antibodies and the size of skin reactions following each of a series of injections of *Pneumococcus*, Type I.

bodies in the serum was made in a large number of animals, but for the sake of brevity, the observations made on 8 rabbits only will be given in detail as typical. Four rabbits received intracutaneous injections of suspensions of heat-killed *Pneumococcus* Type I, and four received similar injections of suspensions of *Pneumococcus* Type III. The size of the reaction at the site of each of the injections was recorded, and the type-specific and species-specific titre of the blood was determined in each animal each week. In Figure 2 (Type I *Pneumococcus*) and Figure 3 (Type III *Pneumococcus*) composite curves indicating the increase of skin reactivity and the development of each kind of antibody during the period of the experiment are given. In each instance, it is seen that during the early injections, the skin reactivity

increased rapidly, while species-specific antibodies appeared in the blood only slowly. With the later injections and the approach of the height of the skin reactivity, the content of the serum in species-specific antibodies increased; later although the skin reactivity remained constant or decreased, nevertheless, the species-specific antibodies increased rapidly. It has not been possible, therefore, to demonstrate any direct relationship between the appearance of species-specific antibodies in the blood and the development of heightened skin reactivity.

Previous experiments have indicated that the degree of active resistance which develops following intracutaneous injections also increases

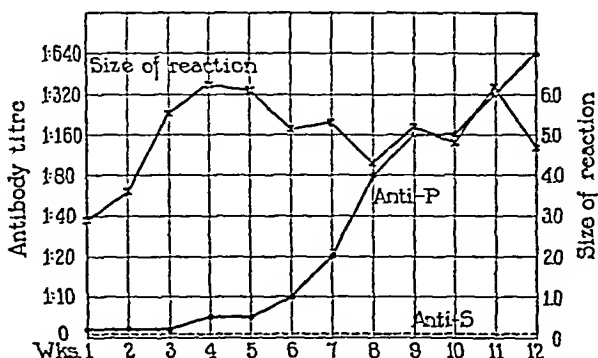


FIGURE 3. The development of antibodies and the size of skin reactions following each of a series of injections of *Pneumococcus*, Type III.

with the number of injections. It seems possible, therefore, that although a relationship may exist between these two phenomena, the exact nature of this relationship is, at the present time, obscure.

#### *Passive Transfer of Increased Skin Reactivity*

Serum obtained from rabbits at the height of skin reactivity was injected intravenously into normal rabbits in quantities varying from 10 to 30 cc. Other normal rabbits were given serum from rabbits which had received repeated intracutaneous injections of egg albumin or *Pneumococcus* "nucleoprotein." Twenty to twenty-four hours following the transfers, injections of suspensions of heat-killed pneumococcus were made into the skin of all the animals which had re-



ceived the serum. Control normal rabbits which had received no serum were also given similar intracutaneous injections. It was found that the rabbits which had received serum from highly reactive animals, those which had received serum from the animals immunized to proteins, and the normal rabbits all exhibited skin reactions of the same character and the same degree of intensity. It seems, therefore, that the property of increased skin reactivity cannot be transferred to the normal rabbits by intravenous injection of serum from the highly reactive animals.

### *The Secondary Reaction*

Andrewes, Derick, and Swift (3) have shown that when rabbits are injected in the skin with live or dead bacteria, particularly with certain strains of *Streptococcus viridans*, a local reaction occurs. This begins to retrogress in about 48 hours, but in over half of the animals, about the eighth or ninth day, a recrudescence of the reaction takes place. Among other observations, they showed that when living R pneumococci, derived from a Type I strain, were injected, secondary reactions occurred in 2 of 13 rabbits tested. When the same organisms killed by heat were injected, or when heated S pneumococci of Type I were injected, a secondary reaction occurred in 2 of 15 rabbits.

In the present study secondary reactions have been observed in 31 out of 54 rabbits which received intracutaneous injections of suspensions of heat-killed pneumococci.

The secondary reaction occurred only after the first injections, never following subsequent ones. The secondary reaction after injections of pneumococci usually appears 7 to 12 days after the injection, and usually after the primary reaction has entirely disappeared. The secondary reaction persists from a minimum of 3 days to a maximum of 25 days, the average duration being 12 days. In appearance it usually resembles the primary reaction, being circumscribed, slightly elevated, pink to reddish in color, with little or no edema, and the lesion has never been observed to break down. Secondary reactions have not been observed following the intracutaneous injections of soluble derivatives of pneumococcus cells.

No relationship could be established between the appearance of secondary reactions and the appearance of type-specific or species-specific antibodies in the blood, nor was there any apparent relationship between the appearance of secondary reactions and the development of active immunity.

In the rabbits mentioned in the previous experiment which had received serum from animals highly skin reactive to pneumococci or from animals which had previously received injections of solutions of egg albumin or "nucleoprotein," secondary reactions following the intracutaneous administration of pneumococci were observed in the same frequency as those occurring in untreated animals.

#### DISCUSSION

The repeated injections of suspensions of heat-killed pneumococci into the skin of rabbits stimulate the development of an increased reactivity of the skin as is shown by the greater size and intensity of the reactions to subsequent injections, and by the fact that doses of heat-killed pneumococci, which in the normal animal are too small to produce any reaction, in the animals which have been previously injected produce marked reactions. The height of increased skin reactivity is reached when 4 to 6 intracutaneous injections have been made, after which the reactivity diminishes. This increased skin reactivity develops after the intracutaneous injection of pneumococci of any type or form, and the reactions are similar, no matter what type or form of *Pneumococcus* has been employed. There is apparently no relationship between the development of an increased skin reactivity and the appearance of type-specific antibodies in the blood, nor does the development of heightened skin reactivity seem to bear any definite relationship to the appearance of species-specific antibodies. While increased skin reactivity appears at a time when increased resistance to infection is present, the relationship between these two phenomena is still obscure.

The heightened skin reactivity is probably dependent upon some alteration in the tissues themselves, since transfers of the blood serum of highly reactive animals to normal rabbits does not endow the latter with the property of reacting with increased intensity.

Finally, it has been found that a secondary reaction may occur following the primary reaction. This is similar to the reactions following the intracutaneous injections of dead or living cells of *Streptococcus viridans* as studied by Swift and his associates (3, 4, 5). With pneumococci this secondary reaction only occurs following the first injection, never following the subsequent ones.

## SUMMARY AND CONCLUSIONS

1. Following repeated intracutaneous injections of heat-killed pneumococci rabbits acquire an increased skin reactivity.

2. The increased skin reactivity reaches a maximum after 4 to 6 injections have been made, after which it becomes greatly diminished.

3. The relationship of increased skin reactivity to active resistance to infection by *Pneumococcus*, and to the presence of species-specific antibodies in the blood, is still obscure.

4. The increased skin reactivity is not transferable by serum from a highly reactive to a normal rabbit.

5. After regression of the reaction to the first injection of *Pneumococcus* into the skin, there frequently follows a recrudescence, or exacerbation, of the reaction.

6. The increased skin reactivity and secondary reactions are incited alike by all types and all forms of *Pneumococcus*.

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# THE IMMUNOLOGICAL SIGNIFICANCE OF COLOSTRUM

## I. THE RELATION BETWEEN COLOSTRUM, SERUM, AND THE MILK OF COWS NORMAL AND IMMUNIZED TOWARDS *B. COLI*

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The passing on from one generation to another of endemic infectious agents and parasites takes place most frequently at birth or soon after. In some instances the egg is the carrier or intrauterine transmission occurs. The mortality of the young generation is governed largely by the variety, concentration and virulence of the infectious agents and parasites domiciled in the herd or flock. The intrauterine transmission of antibodies towards certain enzootic diseases developed in the female parent and carried in the blood occurs in certain species. In others, such as the bovine species, the temporary protection of the young is accomplished by the storage of antibodies in the udder, quiescent before parturition, and the passage of these through the mucosa of the digestive tract when colostrum is taken during the first, and perhaps the second, day of life. The protection afforded by colostrum is probably limited to certain common, ubiquitous species of bacteria living in the digestive tract and on the mucosa of the respiratory tract. The more formidable invaders, such as tubercle bacilli, may be held off temporarily but no complete immunity is afforded, such as that against pathogenic *B. coli* races. In fact certain pathogenic bacteria tend to parasitize the udder. To prevent the newborn calf's ingesting such infectious agents, efforts have been made to pasteurize colostrum or to replace it with milk, frequently with disastrous results. The importance of colostrum in controlling the early mortality due to *B. coli* has been brought forward in earlier publications.<sup>1</sup> At the same time the possibility of substituting normal cow serum for colostrum

<sup>1</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, 36, 181.

was indicated in certain experiments on calves.<sup>2</sup> The use of serum was based on the assumption that it contains at least as much antibody as the colostrum. The experiments to be reported do not wholly substantiate this assumption. In fact the evidence to be presented indicates a much higher concentration in the colostrum than in the serum.

The antibody content towards *B. coli* was singled out, partly because the early diseases of calves are due to members of this group, partly because the antibodies are quantitatively measurable in guinea pigs.

A single strain of *B. coli*, already described,<sup>3</sup> was used in all experiments. This readily mutating strain was employed in the original or (a) form. Several cows were treated subcutaneously and intravenously with living and heated culture suspensions for variable periods of time to furnish a protective serum for control studies.<sup>4</sup> The quantitative estimation of protective antibodies towards *B. coli* was made on guinea pigs weighing 350–400 grams. Exactly 24 hour bouillon cultures were used. The bouillon from the same lot of veal was stored in full bottles in a refrigerator and only this was employed. The minimum fatal dose was determined and  $1\frac{1}{4}$  to  $1\frac{1}{2}$  times this dose was mixed with the serum or colostrum to be tested and injected after 15 minutes into the peritoneal cavity. Control tests of virulence of the culture were associated with each separate experiment.

*The Relative Content of Protective Bodies towards B. coli in Colostrum and in Serum of Untreated Cows.*—The protective action of colostrum having been demonstrated indirectly by withholding this fluid in feeding newborn calves, it seemed desirable to use the test on guinea pigs, described above, to determine quantitatively its protective capacity against the species of bacteria chiefly responsible for the early deaths of calves. The results are given in Table I. To simplify the table and still furnish the information at hand, the amounts of colostrum actually tested are tabulated with the sign (+) indicating protection against the fatal dose, and (—) indicating death of the guinea pig. Lower or higher amounts than those given were not tested. Hence the figures do not in every case represent upper and lower limits. The same strain of *B. coli* (1192a)<sup>3</sup> was used unless otherwise indicated.

Attention is called to certain data in this table. (1) The colostrum of normal cows as a rule contains antibodies to a *B. coli* of the scours

<sup>2</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, 36, 453.

<sup>3</sup> Smith, T., and Bryant, Gladys, *J. Exp. Med.*, 1927, 46, 133.

<sup>4</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1930, 51, 483.

TABLE I  
*The Antibody Content of Colostrum, Milk, and Serum of Normal Cows towards B. coli*

No. of cow	Breed	Source	Entry into herd	Date of test	Dose of			Remarks
					Colostrum (+) = protection	Milk	Serum	
1	Holstein	Wisconsin	Aug., 1926	1926	0.2 (+)	—	—	Milk drawn 7 days later
2	"	Native	—	Oct. 3	0.2 (+); 0.4 (+)	0.4 (-)	0.2 (-); 0.4 (-)	
3	"	"	—	" 3	0.2 (+); 0.4 (+)	—	0.2 (-); 0.4 (-)	Same result with same colostrum refrigerated 7 days
4	Jersey	Oregon	June, 1926	" 15	0.2 (-); 0.4 (-)	—	—	
5	"	"	" 1926	" 19	0.2 (-); 0.4 (+); 0.6 (+)	—	—	
6	Guernsey	Michigan	Oct., 1926	" 19	0.2 (-); 0.4 (+)	—	—	
7	"	Wisconsin	April, 1925	" 24	0.2 (+); 0.4 (+); 0.6 (+)	—	—	
8	Holstein	Native	—	Dec. 3	0.1 (+); 0.075 (-); 0.05 (-)	—	—	
9	"	"	—	" 7	0.1 (+); 0.075 (-); 0.05 (-)	—	—	
10	"	Michigan	Sept., 1922	" 11	0.1 (+); 0.05 (-)	—	—	
11	Jersey	New Jersey	Feb. 2, 1927	1927	0.2 (+)	—	—	Colostrum thin, due to loss on account of overdistension of udder and leakage
12	Holstein	Native	—	" 10	0.1 (-); 0.2 (+)	—	—	
13	"	Michigan	Feb. 27, 1927	Apr. 7	0.2 (-); 0.4 (-)	—	—	
14	Guernsey	Native	—	" 13	0.2 (-); 0.4 (-); 0.6 (+)	—	—	

type. 0.1 cc. to 0.2 cc. may protect guinea pigs against the surely fatal dose. Tests with quantities less than 0.1 cc., when made, were negative. The protective capacity of certain samples was low. Thus No. 4 failed in 0.4 cc. doses, Nos. 5 and 6 in 0.2 cc. doses. To interpret this deficiency, the dates of entry into the herd are given. These cows came in a short time before calving. The inference, based on perhaps too few cases, is that they were not yet immunized to the new flora. On the other hand, No. 1 came within the range of the native cows and the native No. 14 was low. (2) The milk following the colostrum has lost more or less of its protective power, as shown by No. 2. (3) The

TABLE II

*The Relative (Protective) Antibody Content of Serum and of Milk of Two Treated Cows and a Normal Cow*

No. of cow*	Treatment	Serum titer	Milk titer	Agglutination titer of serum towards 1192 <sub>b</sub> (mutant)	Milk Serum (protection)
D	Normal	1.0 (—)	0.8 (—)	—	—
A	Living cultures	0.005 (+)	0.6 (+); 0.4 (—)	1:1,280	$\frac{1}{120}$
B	Heated “	0.005 (+)	0.6 (+); 0.4 (+); 0.2 (+); 0.075(—)	1:2,560	$\frac{1}{40}$

\* For data on the treated cows see *J. Exp. Med.*, 1930, 51, 483.

serum of normal cows drawn at the same time that the colostrum was obtained is distinctly below the colostrum in antibody content. Thus the sera of Nos. 2 and 3 failed to protect in 0.4 cc. doses. The colostrum protected in 0.2 cc. doses and probably in less. Earlier, fairly numerous tests of normal cow sera failed to show any protection in doses of 1 and even 2 cc. Hence tests of normal sera were omitted with exceptions noted.

*The Relative Antibody Content of Serum and Milk of Immunized Cows.*—A number of tests were made to determine the protective titer of the milk of immunized cows. In both cows treated with living and heated cultures, respectively, the milk titer rose in the first to 0.6 cc.,

and in the second to 0.2 cc. Owing to the fact that the original type of *B. coli* used failed to become agglutinated in the immune sera except in very low dilutions, the mutant was used in agglutination tests. Since the immunized cows were not bred, no colostrum from them was available.

*The Effect of Later Feedings of Immune Serum on the Antibody Content of the Blood.*—The significance of lactation on the transfer of immune bodies after the two first days of life has not been satisfactorily cleared up in the earlier experiments. The extensive literature on this subject is more or less contradictory, probably due to the use of different species of animals and alien serum. In a former article<sup>5</sup> the absorption of antibodies in the form of *B. abortus* agglutinins from the digestive tract of calves was prompt and abundant when fed in immune cow serum during the early hours of life. One calf fed a serum of high titer when  $3\frac{1}{4}$  days old showed no increase of agglutinins thereafter. It was furthermore shown that the feeding of milk of high titer was less favorable to the absorption than either colostrum or serum.

The two following cases are further contributions to this subject. Homologous serum was fed to calves  $2\frac{1}{4}$  and 18 days old, respectively. In neither animal was an increase in agglutinins demonstrated as a result of the feeding.

*Calf 1430.* Holstein bull calf, weighing 50–55 lbs., born April 1, 5.30 a.m. Weak and unable to stand at first. Held up to the udder for the first meal when 3 hours old. Left with dam until April 3, 2 a.m. At 10.50 a.m., when about  $2\frac{1}{4}$  days old, it was fed with immune serum of Cow B (see Table II) mixed with milk from the same animal. It drank about 350 cc. of serum. At 7.40 p.m. the calf drank about 250 cc. of serum diluted with milk. Animal thereafter fed milk drawn from the udder of the dam.

April 26. Calf has been normal throughout excepting for a short period of soft, whitish fecal discharges a week ago. The agglutinin titer of the calf's serum before and after the feeding of the immune serum is given in Table III. For comparison a sample of the dam's serum is included.

It will be noted that the dam's colostrum had already given the calf's serum a considerable amount of agglutinins towards *B. coli* 1192<sub>b</sub>. The feeding of immune serum later failed to raise the titer. The serum titer of the dam was only a little higher than that of the calf.

<sup>5</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1923, 37, 671.



The explanation of this slight difference may be looked for in the high concentration of *B. coli* antibody in the colostrum.

In view of the relation existing between the protein output of the urine and the intake of colostrum during the first days of life<sup>6</sup> the protein content of the urine was taken into consideration. When the calf was 29 hours old and before the serum feeding the protein in the urine was sufficient, when the heat test was applied, to produce a coagulum deposit after 24 hours sedimentation equivalent to 15.3 per cent of the volume of the entire fluid. Fourteen hours after the second

TABLE III

*Titration of Serum of Calf 1430 against B. coli 1192<sub>b</sub> after Ingesting 600 Cc. Serum of Titer 1:2,560*

Date of collection of serum	Serum dilutions						
	1:10	1:20	1:40	1:80	1:160	1:320	1:1,280
April 3, 10.50 a.m. (just before feeding serum)	C	C	C	++++	+++	+	-
April 3, 7.40 p.m. (just before 2nd feeding of serum)	C	C	C	++++	+++	++	±
April 4, 9.05 a.m.	++++	++++	++++	+++	++	+	-

*Titration of Dam's Serum*

April 4, 9.10 a.m.	C	C	C	C	+++	++	-	-
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feeding of serum, when the calf was 3 days and 4 hours old, the deposit was only 5.3 per cent, and on the following day it was 1.3 per cent. These figures do not present any evidence that any excess of protein had been absorbed from the serum fed.

Table IV shows the absence of any protection transmitted by the immune serum to the calf's serum.

*Calf 1412.* Fed serum and milk of untreated Cow 1229 at start, with only one discharge of fluid feces on 2nd day. When 18 days old, it was fed 1,000 cc. of

<sup>6</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1924, 39, 303.

immune serum of Cow A (see Table II) administered in 3 doses of 500, 300, and 200 cc. respectively, in one day. On the following day blood was withdrawn for tests 24 and 30 hours after the first feeding of serum, and 12 and 18 hours after the third or last feeding. Neither sample showed any appreciable difference in its agglutinin content from the sample drawn 5 days before the feeding. The urine of

TABLE IV

*Protective Titer of Serum of Calf 1430 Before and After Ingestion of Immune Serum*

Guinea pig No.	Dose of <i>B. coli</i> culture	Dose of serum	Result
	cc.	cc.	
1	0.04	—	Dead in 9 hours
2	0.044	—	" " 8 "
3	0.044	1.0 (a)*	" " 6 "
4	0.044	0.5 (b)	" " 9 "
5	0.044	1.0 (b)	" " 8 "
6	0.044	0.5 (c)	" " 6 "
7	0.044	1.0 (c)	" " 8 "

\* (a) calf serum drawn immediately before feeding; (b) 9 hours after first and just before second feeding; (c) 13 hours after second feeding.

TABLE V

*Calf 1416*

*Agglutinin Titer of Serum before and after Newborn Calf Had Been Fed Serum of Cow B (Heated Cultures) and Normal Milk*

Blood drawn	Serum dilutions								
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Feb. 1, 5.45 a.m.	++	+	+	++	—	—	—	—	—
" 2, 3.10 p.m.	+++	+++	++++	++++	++	+	—	—	—
Serum of Cow B	C	C	C	C	C	C	C	++++	++++

the two following days was as before the feeding. It showed only a very faint clouding after the heat test. Tests on guinea pigs did not indicate any increase in resistance referable to the serum.

Contrasting with these cases is that of another calf (No. 1416, Table V) fed immune serum in place of colostrum during the 1st day of life.

The agglutinins towards *B. coli* (b) were tested in the calf's serum before and after the feeding of immune serum. Serum drawn before the first meal produced a slight deposit of clumps in 1:10 dilution. Twenty-one hours after the first meal the clumping was nearly complete at 1:80. There was a trace at 1:320. The immune serum itself showed nearly complete clumping at 1:2,560. Blood drawn 26 days later had declined in agglutinin content from 1:160 to 1:20.

The protective power of the same samples of serum was distinctly increased by the feeding. The sample drawn before the first meal failed to save the life of the guinea pigs in 1 cc. doses, whereas the sample following the feeding protected in 0.5 cc. but not in 0.25 cc. doses. The serum drawn 26 days later did not protect in a 0.75 cc. dose.

TABLE VI

*Calf 1416*

*Protective Power of Serum before and after Calf Had Been Fed Serum of Cow B*

Dose of <i>B. coli</i>	Dose of serum	Result
cc.	cc.	
0.036	—	Dead in 6 hours
0.04	—	" " 8-9 "
0.04	1.0 (a)*	" " 8-9 "
0.04	0.5 (b)	Lives (40 gram loss in 3 days)
0.04	1.0 "	" (30 " " " 3 " )
0.04	1.0 (a)	Dead in 6 hours
0.04	0.25 (b)	" " 8-9 "
0.04	0.5 "	Lives (no loss in 2 days)

\* (a) = serum before feeding; (b) after feeding.

In another calf (1431)<sup>7</sup> fed serum on the 1st day the agglutinin titer rose from /20 to /160. Eleven days later the titer had receded considerably. One cc. of the serum drawn before the first meal failed to save the life of a guinea pig. Serum drawn 23 hours later protected in 1 cc. but not in 0.5 cc. doses. In still another calf (1417) treated in the same way the agglutinin titer rose from /20 to /80 and the protective dose from above 1 cc. to 0.5 cc. On the other hand, in a third calf (1437) neither agglutinin nor protective antibody increase was detected. The calf, however, continued normal until killed when 1½ months old.

Another method of determining the end period of effective colostrum feeding is to postpone it. Here the danger of early invasion by in-

<sup>7</sup> These figures refer to calves in the second paper of this series, *J. Exp. Med.*, 1930, 51, 486.

fectious agents must be taken into consideration. In two calves reported upon elsewhere<sup>8</sup> the feeding of colostrum delayed 12 and 18 hours, respectively, was not followed by abnormal conditions during the 2 months' life of the calves.

#### SUMMARY

The protective antibody content of normal cow serum is below that of colostrum of the same animal. The method used does not permit the titration of the actual amount of the antibody in serum. Quantities up to 2 cc. have no protective effect. The same limitations apply to the titration of milk owing to the introduction of large quantities of foreign protein into the peritoneal cavity of the guinea pig. When cows were immunized and a serum of high titer obtained, the antibodies in the milk of such cows rose to within the range of the method of testing. The relation of the protective capacity of serum to that of milk was approximately  $1\frac{1}{16}$  and  $\frac{1}{16}$  in the two animals. These figures do not differ much from those obtained by early investigators titrating the antitoxic content of serum and milk of animals undergoing immunization with diphtheria toxin. In the two experiments on calves,  $2\frac{1}{4}$  and 18 days old respectively, fed a highly protective serum, no increase in agglutinins or protective antibodies could be demonstrated. The postponement of colostrum to the 12th and 18th hour, respectively did not prevent normal growth.

<sup>8</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1930, 51, 488.



## THE IMMUNOLOGICAL SIGNIFICANCE OF COLOSTRUM

### II. THE INITIAL FEEDING OF SERUM FROM NORMAL COWS AND COWS IMMUNIZED TOWARDS *B. COLI* IN PLACE OF COLOSTRUM

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It has been shown that in place of colostrum normal cow serum fed and injected will protect most calves so treated against the various early diseases associated with *B. coli*. It has also been shown that in normal cow serum the specific protective bodies against *B. coli* are much weaker than those in colostrum and below the range of values detected by the intraperitoneal injection of mixtures of cultures and serum into guinea pigs.<sup>1</sup> It may be assumed that colostrum, and cow serum in much less degree, contain antibodies towards other endemic infectious agents which are held in more or less complete subjection in older animals. Hence the feeding of a serum containing a high concentration of *B. coli* antibodies might not protect to the same degree against other diseases developing later and more slowly. However, it was thought best in carrying out the following experiments to try the serum of cows hyperimmunized towards *B. coli*, to keep the serum monovalent, and to administer it only by mouth in full imitation of the natural intake of colostrum. Calves were also fed with normal cow serum. Methods for testing the relative protective power of the sera against *B. coli* on guinea pigs are given in another paper.<sup>1</sup>

*The Immunization of Cows.*—To provide the immune serum cows were treated in several different ways. *B. coli* strain 1192<sub>a</sub><sup>2</sup> was chosen. This was a highly virulent, hemolytic race, not fermenting saccharose. The cows received both intravenous and subcutaneous injections variously spaced in time according to the condition of the animal. One cow was treated with living cultures, another with

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<sup>1</sup> Smith, T., *J. Exp. Med.*, 1930, 51, 474.

<sup>2</sup> Smith, T., and Bryant, G., *J. Exp. Med.*, 1927, 46, 133.

cultures heated at 62°C. for 30 minutes, a third received heated cultures of the mutant designated 1192<sub>b</sub>, and a fourth bouillon culture filtrates. All intravenous injections were followed by more or less prompt reactions on the part of the respiratory system similar to the disturbances produced in calves by the intravenous injection of filtrates.<sup>3</sup> To avoid too great depression as a result of intravenous injections, the subcutaneous route was frequently used. Large local swellings appeared with or without a febrile reaction, which always followed intravenous administration. There was evident a tendency towards hypersensitiveness when the treatment had progressed 6 or more months. The doses then had to be reduced accordingly.

Table I gives a few data concerning these treated cows. The protective titer of the sera as tested on guinea pigs was about the same for

TABLE I  
*Immunization of Cows to B. coli*

No. of cow	Breed	Strain of <i>B. coli</i>	Treatment	Treatment begun	Treatment terminated	Protective titer of serum
						cc.
1109	Guernsey	1127 <sub>a</sub>	Living culture	Nov., 1924	April, 1925	0.1
A	Holstein	1192 <sub>a</sub>	" "	" 1925	July, 1927	0.005
B	Jersey	1192 <sub>a</sub>	Heated "	Sept., 1926	" 1927	0.005
C	Guernsey	1192 <sub>b</sub>	" "	Dec., 1927	March, 1928	0.02*
D	"	1192 <sub>a</sub>	Culture filtrate	Nov., 1926	June, 1927	0.5

\* Towards mutant.

the cows treated with living and heated cultures respectively. The 48 hour bouillon filtrates failed to produce a potent serum. The serum of the cow treated with heated cultures of the *B. coli* mutant had little or no protective power over the original or (a) form but was quite effective in presence of the mutant.

*The Feeding of Newborn Calves with Serum from Normal and Immunized Cows in Place of Colostrum.* The method adopted for handling the calves selected for the serum treatment did not differ appreciably from that used in earlier work.

The calf was taken from the dam immediately after birth to the warmed Department units and thoroughly dried by rubbing with towels. Every effort was made to keep the animal protected from undue exposure.

<sup>3</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1927, 46, 123.

The serum was fed from a bottle, provided with a nipple, in three doses, each time mixed with milk. In the first and the second group, the first dose, given as soon as possible and usually within an hour after birth, was about 400 cc. serum. The second dose of 200 cc. was given about 6 to 7 hours later, and the third of 100 cc. from 6 to 10 hours after the second. The total amount of serum, 600 to 700 cc., was thus fed within the first 18 hours of life. In the third group larger amounts were given, also in 3 doses. Thereafter milk from cows well along in lactation was fed 3 times a day, at first from bottles and then from a pail. After 4 weeks some hay and grain were added to the ration.

For agglutination and protection tests blood was withdrawn from a jugular by piercing it with a hollow needle and allowing the blood to run into sterile containers. The tests were made as described in another article.<sup>1</sup>

Urine was obtained whenever possible either when passed voluntarily or by gentle manipulation of the external genitals. The examination was limited to the following operations. The deposits of cloudy fluid thrown down with the centrifuge were examined microscopically. Cloudy samples were filtered until clear. The clear urine was slightly acidified with acetic acid, brought to boiling in a graduated centrifuge tube, and the bulk of any coagulum which had formed measured after 24 hours sedimentation. This quantity divided by the total volume of urine in the graduate is given as per cent protein precipitate. There were also recorded the specific gravity, color, and reaction to litmus. The temperature was taken twice daily. Most of these data have not been reproduced in this paper.

The experiments were carried out on 3 groups of calves in 3 successive years. The gross results are given in Table II, III, and IV. Each group will be briefly discussed and the results summarized.

*First Group (1926-7).*—Two calves fed milk from an immune cow both succumbed within a few days after birth. The lesions indicated *B. coli* septicemia associated with unrestricted multiplication of *B. coli* in the small intestine. Nine calves which were fed serum of cows treated with living and dead *B. coli* cultures lived beyond the scours period without manifesting any appreciable disturbance. The serum of the fed calves in nearly all cases contained a demonstrable increase of *B. coli* agglutinins and antibacterial substances. Of three calves receiving serum of an untreated cow, one died on the 9th day. One calf receiving serum of the filtrate-treated cow (D) survived, the other died when 4 days old.

Subsequent happenings in calves kept until at least a month old presented new problems. Two manifested symptoms referable to disturbances of the central nervous system. One of these and three others developed a renal disease differing essentially from that described as the "white-spotted kidney" due to *B. coli*.<sup>4</sup> Two were killed too soon (12 and 21 days respectively) to warrant the inference that

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<sup>4</sup> Smith, T., *J. Exp. Med.*, 1925, 41, 413.



TABLE II

First Group. Calves Fed Serum From Normal and Immunized Cows (600-700 Cc.)\*

No.	Breed and sex	Date of birth	Weight at birth lbs.	Fed serum of cow No.	Weight at death lbs.	Result
1369	Guernsey male	1926 May 20	55	(Milk only of A**)	—	Dead in 2 days. <i>B. coli</i> septicemia.
1396	" "	Nov. 24	—	Normal serum	—	" " 9 " Hemorrhages in 4th stomach.
1401	Holstein "	Dec. 2	90	" "	92	Killed in 12 days. Slightly fatty liver and renal congestion.
1403	" female	" 7	100	A	127	Killed in 21 days. Normal.
1405	" male	" 8	85	A	149	" " 34 " "
1408	" "	" 21	100	A	—	Sold " 23 " "
1411	" "	1927	70	D***	165	Killed in 77 days. Renal lesions.
1412	" female	Jan. 2	70	Normal serum	—	Used in another experiment when 34 days old.
1416	" "	Feb. 1	70	B****	—	Killed when 97 days old. Renal lesions. Blindness.
1417	Guernsey "	" 4	80	B	—	Killed when 97 days old. Normal.
1418	Holstein "	" 9	70	A	94	Dies when 30 days old in convulsions.
1421	Jersey male	March 1	50	D	—	" in 4 days. Hemorrhages in 4th stomach. Scours.
1423	Holstein "	" 3	85	(Milk only of A)	—	Dies in 5 days. Scours. Peritonitis.
1430	" "	April 1	50	(Control)	214†	Killed when 96 days old. Normal.
1431	" "	" 6	85	B	210	" " 75 " "
1434	Guernsey female	" 13	40	A	56	Moribund when 44 days old. Hemorrhages in 4th stomach. Renal lesions.
1437	" "	May 5	70	A	130	Killed when 2 months old. Normal.
1449	" male	" 27	90	(Control)	137†	" " 1 month " "
1450	Holstein "	" 27	85	"	138†	" " 6 weeks " "

\* The 3 first calves received 600 cc., the rest 700 cc.

\*\* Cow treated with living *B. coli*.\*\*\* Cow treated with *B. coli* filtrates.\*\*\*\* Cow treated with heated *B. coli*.

† 9 days before slaughter.

TABLE III  
Second Group. Calves Reared Normally or Serum-Fed

No.	Breed and sex	Date of birth	Approximate weight at birth lbs.	Fed serum of cow	Weight at death lbs.	Result
1461	Holstein male	1927 Sept. 6	119	(Control)	171	Killed, 1 mo. 17 days old. Normal.
1466	Jersey "	" 21	40	B	134	" 2 " 8 " "
1467	Guernsey "	" 21	76	(Control)	184	" 2 " 23 " "
1469	" "	" 28	80	A	114	Dies when 43 days old in convulsions.
1470	Holstein "	" 28	85	B	220	Killed, 2 mo. 15 days old. Normal.
1473	Guernsey female	Oct. 28	75	(Control)	150	" 2 " 8 " "
1477	" "	Dec. 12	70	A	142	" 1 " 25 " "
1478	" male	" 12	60	A	—	" 2 " 1 day " "
1479	Holstein "	" 13	85	(Control)	149	" 1 " 17 days " "
1480	" "	" 28	80	B	181	" 1 " 30 " "
1485	Guernsey "	1928 Jan. 10	65	(Control)	141	" 2 " 2 " "
1492	Jersey "	Feb. 13	55	A	135	" 1 " 27 " "
1493	Holstein female	" 17	80	A	171	" 1 " 30 " "
1494	" male	" 14	85	(Control)	208	" 2 " 9 " "
1498	" "	March 14	85	(Milk only of B)	91	Dies in 37 hours. <i>B. coli</i> septicaemia.
1499	Guernsey "	" 15	60	A	92	Killed, 2 mo. old. Scours. <i>B. coli</i> cystitis.

TABLE IV  
*Third Group. Calves Reared Normally or Serum-Fed*

No.	Breed and sex	Date of birth	Ap- proximate weight at birth	Cow serum fed and amount	Weight at death	Result
		1928	lbs.		lbs.	
1511	Guernsey male	May 22	—	C, 700 cc.	140	Dies when 1 mo. 28 days old. Renal lesions.
1513	Holstein "	Aug. 16	80	(Control)	158	Killed " 1 " 15 " " Normal.
1514	Guernsey "	Oct. 1	65	C, 700 cc.	133	" " 2 " 2 " " "
1516	" "	" 3	60	C, 700 cc.	112	Sold " 2 " 4 " " Clinically normal.
1518	" "	" 4	55	(Control)	113	" " 2 " 3 " " " "
1522	Holstein "	Nov. 4	85	"	202	Killed " 2 " 1 day " Normal.
1529	" female	Dec. 12	85	Normal serum, 1400 cc.	156	" " 2 " old. Spotted kidney.
		1929				
1531	" male	Jan. 4	110	(Control)	164	" " 2 " 4 days old. Normal.
1532	" female	" 11	75	Normal serum, 700 cc.; D, 700 cc.	147	" " 2 " 8 " " Slight renal lesions.
1533	" male	" 12	95	A, 145 cc.; C, 1260 cc.	172	" " 2 " 3 " " Normal.
1542	" female	Feb. 11	75	Milk only	120	Dies " 1 " 22 " " Large abdominal abscess.
1549	Guernsey male	March 7	75	(Control)	160	Killed " 2 " 16 " " Normal.
1553	Holstein female	" 19	100	Colostrum de- layed 18 hr.	190	" " 2 " 1 day " Intestines congested.
1554	" male	" 20	85	Normal serum, 700 cc.	170	" " 2 " 3 days old. Normal.
1556	" "	April 8	85	Colostrum de- layed 12 hr.	185	" " 2 " 4 " " "
1562	Jersey female	" 15	45	Normal serum, 700 cc.	93	" " 1 " 27 " " "

they would have remained normal indefinitely. Three of the calves had hemorrhages of the mucosa of the fourth stomach. This condition, to which attention has been called in previous publications, is probably the result of prenatal causes not yet defined. The hemorrhages vary from pale red dots to active petechial, closely set hemorrhages covering the entire mucosa. They may disappear quickly or continue to discharge blood for weeks and are probably the immediate or remote contributory cause of death in such animals. They may have been responsible for the death of Calf 1434. If we exclude this animal, there is one tardy death among 6 fed with serum of Cow A and one calf with renal disease among 3 fed with serum of Cow B.

Three control calves kept with their dams for a few days and then put under the same conditions as the experimental calves remained normal.

*Second Group (1927-8).*—In planning the experiments of the first group, the possibility that calves which had successfully weathered the early dangerous *B. coli* period might suffer from diseases developing later was not fully realized. Some calves were killed too early. The pathological conditions observed in the first group and in earlier published studies led to various conjectures as to the nature and causes of the conditions. To eliminate some of these a second group was subjected to the same treatment and a few more controls were introduced. These remained with the dam from 4 to 6 days and were then transferred to the Department units. This group was kept under observation long enough to permit the evolution and closer study of the diseases which had appeared in the first group. All serum-fed calves received 700 cc. serum.

From Table III it will be noted that there were 6 controls, 9 serum-fed and 1 milk-fed calf. Those surviving were kept at least 1½ months and most of them 2 months and longer. At the end of this period they were killed and autopsied. All controls remained normal. Of the serum-fed animals, one died in convulsions when 43 days old. One (1499) went through several attacks of scours and later developed a *B. coli* cystitis. The calf receiving milk only from Cow B died of *B. coli* septicemia.

*Third Group (1928-9).*—In this group were 16 calves. The treatment was the same as in the other groups, excepting that the amount and source of the serum fed differed from those of the earlier groups and from animal to animal. Five calves were introduced as controls. Three were fed with serum of Cow C (mutant *B. coli* treatment), and 3 received normal cow serum. One received normal serum and serum of Cow D (*B. coli* filtrate serum) in equal amounts; another chiefly C serum plus 1/10 A serum. In two the first feedings were with milk and colostrum feeding was delayed 12 hours and 18 hours respectively. One was fed milk only. This animal lived nearly 2 months and died within 15 minutes in convulsions. The autopsy showed the presence of a large abdominal abscess, originating in the track of the urachus. All 5 controls remained normal. Of the serum-fed, one died with renal disease. A second, although with normal

clinical history and autopsy, had slightly heavier kidneys and intranuclear bodies in the uriniferous tubules to be described more fully in another paper.<sup>5</sup>

### DISCUSSION

The outcome of the three experiments shows that feeding an adequate amount of serum (600 cc. or more), either [from normal cows or from those immunized with one type of *B. coli*, in place of colostrum prevented the early fatalities attributable to *B. coli*, but did not protect all calves from renal lesions or fatal convulsive attacks during the 2nd month of life. That the serum is somehow negatively responsible for these delayed morbid conditions is indicated by the significant fact that all 11 control calves of the second and the third group remained normal whereas of the 17 serum-fed animals of these same groups 2 died. The greater success in preventing disease in these groups as compared with the first group cannot be adequately interpreted in default of knowledge concerning the causes of the convulsive symptoms and renal lesions. The rarity and unexpected appearance of these conditions have interfered thus far with a more thorough study of them.

The age of the serum, normal and immune, varied considerably. Some of it was nearly 2 years old when fed. The guinea pig tests indicated very little antibody decline when the serum was kept refrigerated in nearly full containers. All of the serum was passed through Berkefeld filters and stored without the addition of antiseptics.

The value of the monovalent *B. coli* serum as compared with that of normal cows could have been determined only by a large series of tests on account of the genetically variable animal material, the fluctuating conditions of seasons and of the dairy cow during pregnancy. However, the complete history of the calves, of which the details have been omitted, indicated a greater freedom from early diarrheas among those fed immune than among those receiving normal serum. The experiments bring out the fact that there is nothing qualitatively different in colostrum from normal serum but that the former is quantitatively more effective so far as the content of antibodies is involved. It should be borne in mind that the normally fed calf receives an amount

<sup>5</sup> Smith, T., *J. Exp. Med.*, 1930, 51, 519.

of colostrum much larger than the serum fed. It would require 2 to 3 liters of normal serum to match the antibody content of the usual amount of colostrum ingested. The inference is thus permitted that there is a large factor of safety in the normal feeding of the newborn calf. That colostrum contains favoring substances not present in serum, as has been claimed at times, is not denied, but the experiments indicate that a sufficient quantity of serum is in 9 out of 10 cases capable of replacing colostrum without injury to the calf. The experiments also indicate that the chief and perhaps the only function of colostrum is to protect the young animal against an early invasion of miscellaneous semi-pathogenic microorganisms by way of the digestive and the umbilical tract.

The pathological conditions appearing in the 2nd month in the serum-fed calves are probably not new diseases but such as are occasionally encountered in calves fed naturally. In the practical application of serum feeding to eliminate contact with the dam immediately after birth, it is suggested that the method might be distinctly reenforced by two or three subcutaneous injections of small quantities of serum during the first 24 hours of life.

The advantages of immune serum do not appear to outweigh the cost of production and it is suggested that the normal serum be used, preferably in larger quantities, such as 1200 to 1500 cc. If possible, the source of the serum should be the same herd or at least herds in the same section of the country, until further experiments should demonstrate no important differences between, let us say, serum from the eastern states and that from the middle or western states. The interchange of cattle is, however, of more importance than locality in the dissemination of infectious agents that tend to become endemic.

The feeding of milk alone, even from highly immunized cows, is again shown powerless to prevent rapid or delayed death.

Though the outcome of these experiments appears on the surface of little practical value, it may be safely predicted that the encroachment of the slowly developing, insidious infectious agents—such as tuberculosis, contagious pleuro-pneumonia, paratuberculosis, and diseases due perhaps to other still undefined agents—will be more dangerous in the dairy herds of the future as they continue to grow in size. In the thoroughbred Guernsey and Jersey cattle the spread of paratubercu-

losis may eventually compel the use of some procedure which separates the calf from its dam at birth.

#### CONCLUSION

Under certain safeguards, such as isolation, calves from a large dairy herd have been raised by feeding normal and immune cow serum in place of colostrum. The losses were about one out of ten in the later experiments. This outcome may probably be improved by the subcutaneous injection of serum during the first day. This loss may be no greater than that under ordinary conditions, since sporadic deaths among calves are not infrequent. However, no satisfactory statistics are available for comparison with results as given above.

# THE RELATIONSHIP OF YELLOW FEVER OF THE WESTERN HEMISPHERE TO THAT OF AFRICA AND TO LEPTOSPIRAL JAUNDICE\*

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In the spring of 1928 the accumulated evidence concerning the etiology of yellow fever presented seeming discrepancies which suggested that more than one independent disease had been investigated as yellow fever. To help overcome this confusion the International Health Division of the Rockefeller Foundation undertook to bring together in one laboratory the yellow fever viruses of West Africa and South America and to determine their relationship. The study was begun in June, 1928, at The Rockefeller Institute for Medical Research.

The conflicting evidence referred to may be summarized briefly.

In Cuba in 1900 and 1901 the Yellow Fever Commission of the United States Army under Major Walter Reed (1) found that the etiological agent of yellow fever was present in the circulating blood during the first three days of fever; that it was not cultivable by any of the bacteriological methods used; that it would pass a Berkefeld filter capable of holding back *Staphylococcus pyogenes aureus*; and that it could be readily transferred from a sick person to a well one with the production of infection, by the mosquito *Aedes aegypti*. The bite of even one mosquito was sufficient to cause the disease.

From 1918 to 1924 a leptospira was isolated by Noguchi and other investigators (2) from patients in outbreaks of yellow fever in Ecuador (Guayaquil), Mexico (Mérida and Vera Cruz), Peru (Morropón), and Brazil (Palmeiras). Like the specific agent studied by the commission under Walter Reed this organism was present in the blood early in the disease, it was not cultivable in the ordinary

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culture media for bacteria, and it was able to pass Berkefeld filters (V and N). It seemed to differ, however, in not being easily transferable by *Aedes aegypti*. Noguchi (3) found that he could infect guinea pigs with this leptospira, which he named *Leptospira icteroides*, by means of the bites of *Aedes aegypti* which had fed previously on a patient or on infected guinea pigs, but that transmission was obtained infrequently considering the number of mosquitoes employed. Another difficulty in the way of accepting the organism as the causative agent of yellow fever was the uncertainty of the diagnosis in the cases in which leptospirae were isolated. In the individual case, infectious jaundice, caused by *Leptospira icterohaemorrhagiae*, is ordinarily indistinguishable from yellow fever on the basis of symptoms. Elliott (4), who was associated with Noguchi in 1918 as clinician of the commission which studied yellow fever in Guayaquil, held the following opinion: "Clinically yellow fever is similar to infectious jaundice. The differences existing between the two diseases appear to be chiefly those of degree. There is more marked jaundice and less hemorrhage in yellow fever than in infectious jaundice." In the differential diagnosis between these diseases the nature of the prevailing epidemic was of necessity frequently allowed to determine the decision with regard to the individual case. To meet this difficulty Noguchi made comparative studies of *L. icteroides* and *L. icterohaemorrhagiae* and decided (2) that they were distinct morphologically and serologically, and that the evidence appeared to warrant the conclusion that *L. icteroides* is the cause of yellow fever. This view became generally accepted.

In 1925 the West African Yellow Fever Commission of the Rockefeller Foundation undertook the study of a disease in West Africa known as yellow fever and indistinguishable from the yellow fever in South America by clinical or pathological characteristics. As summarized by Stokes, Bauer, and Hudson (5), 67 cases of yellow fever were studied bacteriologically by Muller, Kligler, Sawyer, and Bauer between January, 1926, and May, 1927. Although a large number of cultures were made and many guinea pigs inoculated, no leptospira could be isolated nor any other organism which could have a relation to the disease. Yellow fever in West Africa was obviously not due to *L. icteroides*.

In 1927 it was discovered by Stokes, Bauer, and Hudson (5) that the monkey *Macacus rhesus* is susceptible to yellow fever. Using this monkey as an experimental animal they found that the specific agent of the yellow fever of Africa resembled that of the classic yellow fever studied by the army commission in Cuba in being present in the blood early in the disease, in not being cultivable, in filtrability, and in ease of transference by means of *Aedes aegypti*.

In 1928 Aragão (6) transferred the yellow fever of South America from patients to *Macacus rhesus* monkeys during an epidemic in Rio de Janeiro. The infectious agent was found to be filtrable and was easily transmitted by *Aedes aegypti*. A search for *L. icteroides* in 15 cases of yellow fever yielded negative results.

In the meanwhile several experimenters made observations which called in question the opinion that *L. icteroides* was the cause of the true yellow fever of

the Americas, as represented by the disease studied by the commission under Reed. Sellards (7) was unable to demonstrate by serological methods any relationship between a yellow fever outbreak in Parahyba, Brazil, and *L. icteroides* or *L. icterohaemorrhagiae*. Theiler and Sellards (8) and Schüffner and Mochtar (9) compared *L. icteroides* and *L. icterohaemorrhagiae* and obtained strong evidence of their serological identity.

Attempts to transfer *L. icteroides* by means of *Aedes aegypti* failed in the hands of a number of investigators (Kligler (10), Gay and Sellards (11), Schüffner and Mochtar (9), and Sawyer and Bauer (12)) and they became convinced that this mosquito is not suited to act as intermediate host for *L. icteroides*.

When our experiments were undertaken there seemed to be need for further investigation of the relationships of the "yellow fever" of West Africa, the "yellow fever" of South America not due to a leptospira, the "yellow fever" of the Americas apparently caused by a leptospira, and a hypothetical "yellow fever" in which the etiological factors of the two last mentioned diseases are both present.

### *Collection of Strains of Yellow Fever Virus*

The strains of virus used in our experiments were the French and the Asibi from West Africa and the F. W. from Brazil.

The French strain was given us by Dr. A. W. Sellards and Dr. Max Theiler of the Harvard Medical School. It had been obtained originally from a yellow fever patient in Senegal by Mathis, Sellards, and Laigret (13). During our experiments this strain was indistinguishable from the Asibi strain in its effects on monkeys.

The Asibi strain was sent us from Lagos, Nigeria, by Dr. Henry Beeuwkes, Director of the West African Yellow Fever Commission of the Rockefeller Foundation. It had been obtained in the Gold Coast from an African native during an attack of yellow fever, and had been used by Stokes, Bauer, and Hudson (5) in most of their experiments.

The establishment in our laboratory of a strain of yellow fever virus from South America proved to be difficult. We are indebted to Dr. F. L. Soper and Dr. H. Muench for obtaining for us a large number of specimens of blood from yellow fever patients in Rio de Janeiro. We wish to acknowledge also our special obligation to Dr. Clementino Fraga, Director of the National Department of Health, and to Dr. Sinval Lins, Chief of the Communicable Disease Section of Hospital São Sebastião, for their courtesy in granting Dr. Soper and Dr. Muench

free access to the patients. Specimens of blood and tissues from monkeys experimentally infected with yellow fever were received through the kindness of Dr. H. de B. Aragão of the Oswaldo Cruz Institute in Rio de Janeiro and of Dr. N. C. Davis and Dr. J. H. Bauer of the Yellow Fever Laboratory of The Rockefeller Foundation at Bahia.

The specimens of human blood from Rio de Janeiro were taken from 103 patients having yellow fever or suspected of having it. A citrated specimen from each patient was received and also dried blood from three and clotted blood from four. Among the patients supplying the blood were at least two from whom yellow fever was transmitted to monkeys in Rio de Janeiro. This was accomplished by blood inoculation by Da Cunha and Muniz (14) in the case of F.W., and through the bites of mosquitoes by Aragão (15) in the case of N.M. The blood was drawn on the first day of the disease from 11 patients, on the second from 37, on the third from 53, and on the fourth from one. In one instance the day of disease was not recorded.

As soon as received, the specimens were injected into *M. rhesus* monkeys, in varying amounts, usually intraperitoneally, rarely subcutaneously. Of the 110 specimens, including duplicates, 16 were injected into separate animals. The remaining 94 were divided into 14 groups and pooled, and part of each of the 14 mixtures was injected into one or two monkeys. In some instances animals which showed no symptoms were inoculated later with another specimen as the number of available monkeys was limited. Test injections with a known virus were not given at the end of the observation period to determine whether immunity to yellow fever had been established as we had no yellow fever virus during the early part of the work. The rectal temperature of the monkeys under observation was taken twice each day.

The results from the inoculation of the specimens of human blood sent from Rio de Janeiro were uniformly negative. Most of the monkeys developed no fever. Six had isolated rises of temperature which seemed of no significance. We concluded that it was not practicable to isolate strains of yellow fever from the blood of patients in South America while working at so great a distance from the source of material.

Attempts to bring in a strain of yellow fever already established in monkeys finally met with success. From Rio de Janeiro Dr. Aragão sent us material from monkeys infected with several strains (F.W., N.M., N.M. and J.K., N.M. and D.A., J.K. and R.M.). We received citrated, glycerinated, and dried monkey blood (one specimen of each) under refrigeration; an emulsion of mosquitoes which had fed on an infected monkey; and monkey liver which had been refrigerated (5 specimens), frozen (3), glycerinated (3), dried (2), and one kept at room temperature. These eighteen specimens were prepared and injected into animals

intraperitoneally or subcutaneously. Six of the animals died from causes other than yellow fever. The other animals remained normal except one.

A monkey which had been inoculated with a specimen of frozen liver, F.W. strain, tenth passage in monkeys, showed fever on the seventh day and recovered. After an observation period of 28 days from the date of inoculation this monkey was given a test injection of African yellow fever virus, Asibi strain, and was found to be immune. The strain obtained by bleeding this monkey at the beginning of its fever was carried through many passages in this laboratory, and the F.W. virus used in our experiments was derived from this succession.

Like the African strains, the F.W. strain thus procured could withstand long storage under suitable conditions. At one time the strain was apparently lost, but was reestablished by inoculating a monkey with stored liver tissue. The specimen of liver had been kept continually frozen for 67 days. Between experiments this strain was successfully preserved in storage for varying intervals up to 92 days in monkey blood dried in the frozen state by the methods used by Sawyer, Lloyd, and Kitchen (16) in preserving African strains of yellow fever virus.\* We have not yet tested older dried blood specimens of the F.W. strain by inoculation of monkeys.

The material from Bahia, Brazil, consisted of 28 specimens of blood and liver from 20 monkeys experimentally infected with yellow fever of the B.B. strain (27 specimens) and the S.R. strain (1 specimen). These strains had been obtained from yellow fever patients in Bahia, Brazil, by Davis and Burke (17). This material was injected into monkeys and the B.B. strain was established in our laboratory, but the virulence of this strain for monkeys was so low that it seemed unsuitable for our projected cross-immunity experiments.

In the hands of Davis and Burke (17) the B.B. strain has shown a higher degree of virulence. Immunity against African virus was possessed by four of eight animals that developed fever after inoculation by us with the B.B. strain specimens. This adds to the evidence supporting Davis (18) in his conclusion that this strain is immunologically the same as African yellow fever virus.

### *Bacteriological Examinations*

The specimens of citrated human blood received from Rio de Janeiro were tested for bacterial contamination by inoculation of broth or agar slants. Only three specimens produced visible growths and they were caused by three different organisms. A more critical bacterial examination was made by Frobisher (19) of 30 of these specimens of human blood and also of the blood of 16 monkeys infected

\* A specimen of African yellow fever virus (French strain) in monkey blood preserved in this way has been tested after one year in storage and found to be highly virulent for *rhesus* monkeys.

with African or South American yellow fever virus and of the liver tissue of 10 monkeys infected with African virus. Cultures were made in a wide variety of media, including the Noguchi leptomedium, and guinea pigs were inoculated. The results were essentially negative.

Of the specimens of citrated human blood sent us from Rio de Janeiro, 66 were examined also by H. R. Muller and E. B. Tilden (20) at The Rockefeller Institute for Medical Research. Two of these specimens yielded cultures of a spiral organism agreeing with the cultures of *L. icteroides* isolated in yellow fever epidemics by Dr. Noguchi. Both of the specimens containing leptospirae came from severe cases with the symptoms of yellow fever; one of the cases was fatal. Both specimens were among the first 36 examined, which group was included in the 103 specimens inoculated by us into monkeys.

*Tests of Monkeys Immunized with American Yellow Fever Virus for Immunity to African Virus*

In order to show the relationship of American yellow fever to the African, monkeys were immunized with American yellow fever virus and afterward tested for susceptibility to African virus, and *vice versa*.

In Table I are given the results of the tests of eleven monkeys which had exhibited fever after intraperitoneal inoculation with the F.W. strain of American yellow fever virus. Monkeys which had not shown fever after inoculation with this strain were sometimes found to be immune to African virus and sometimes not. They were all excluded from this experiment as some had probably not been infected.

Three of the monkeys were inoculated a second time with the F.W. strain from 5 to 7 weeks after the first inoculation, in order to make certain that they had become immune to American yellow fever virus. To obtain additional evidence regarding the immunity produced and a better idea of its degree, blood was taken from six of the animals between the 16th and 41st days after the last injection of American virus, and the serum was tested for protective power against African virus. All eleven of the monkeys were tested by intraperitoneal injection of African virus when at least 30 days had elapsed after their last inoculation with American virus, and the high virulence of the African virus used was shown by its effect on control animals. Except when otherwise stated in the table, the period

of observation after the test inoculation was 30 days or longer. Only temperatures of 40°C. or over were accepted as fever unless otherwise stated.

All of the eleven monkeys immunized against American yellow fever virus survived inoculation with a highly virulent strain of African yellow fever virus, and only one showed fever within the period of

TABLE I

*Immunity to African Yellow Fever Virus in Monkeys after Infection with American Yellow Fever Virus*

Monkey	First inoculation with American virus		Second inoculation with American virus		Protection test of monkey's serum against African virus†	Test of monkey for immunity by inoculation with African virus (Asibi)
	Strain	Febrile period*	Strain	Febrile period*		
B	F.W.	5.5 to 12.0	F.W.	11.0 to 13.0	Protected‡	Immune
C	F.W.	2.5 to 15.0	F.W.	None	Protected	Immune
D	F.W.	2.5 to 9.0	Not inoculated		Protected	Immune
E	F.W.	7.0 & 12.0	Not inoculated		Protected‡	Immune
F	F.W.	7.5 to 9.5§	F.W.	None	Protected	Immune
G	F.W.	3.0§	Not inoculated		Not tested	Immune
H	F.W.	2.0 to 8.5	Not inoculated		Protected‡	Immune
I	F.W.	6.0 to 7.5	Not inoculated		Not tested	Immune
J	F.W.	3.0 to 4.5	Not inoculated		Not tested	Immune¶
K	F.W.	4.0§	Not inoculated		Not tested	Immune
L	F.W.	4.0 to 12.0	Not inoculated		Not tested	Immune

\* Expressed in days after inoculation.

† Performed with Experiment IV; same methods, same virus (Asibi), regular amount of serum, and same controls (Table IV).

‡ Had one elevation of temperature; Monkey B on 14th day after inoculation, Monkey E on 7th, Monkey H on 8th.

§ Maximum temperature 39.7°C. (Monkey G) or 39.9°C. (Monkey K).

|| Observed for 24 days only.

¶ Fever 4.0 to 6.0 day. Observed for 12 days only.

observation. The sera of six of these monkeys taken before the test inoculation were tested for power to protect monkeys against the same African strain. All protected against death, although three permitted fever.

*Tests of Monkeys Immunized with African Yellow Fever Virus for Immunity to American Virus*

As it was a rare exception for a monkey to survive inoculation with fresh virus of the African strains which we used (French and Asibi), it was necessary in our cross immunity tests to use monkeys which

TABLE II  
*Immunity to American Yellow Fever Virus in Monkeys after Infection with African Yellow Fever Virus*

Monkey	First inoculation with African virus		Second inoculation with African virus		Protection test of monkey's serum against American virus (F.W.)	Test of monkey for immunity by inoculation with American virus (F.W.)
	Strain	Febrile period	Strain	Febrile period		
M	Asibi	None	Asibi	None	Not tested	Immune*
N	Asibi	None†	Asibi	1.5	Not tested	Immune*
O	French	None†	Asibi	None	Not tested	Immune
P	French	None†	Asibi	None	Not tested	Immune
Q	French	12.5 to 15.0	Asibi	None	Not tested	Immune
R	French	6.0 to 7.0	Asibi	4.5	Not tested	Immune
S	Asibi	5.0	French	None	Not tested	Immune
T	Asibi	3.0, 3.5	French	None	Not tested	Immune
U	French	1.5 to 5.0	Not inoculated		Doubtful‡	Immune
Control: C§						Febrile period 2.5 to 15.0

\* Had received an injection of the B. B. strain of American virus but, as the control monkey as well as the animal tested showed no reaction, the monkey was reinoculated 14 days later with the F. W. strain.

† Protected by an injection of African immune serum from recovered African natives (Monkeys N, and P) or from immunized Monkey A, Table IV (Monkey O).

‡ Had irregular unexplained rises of temperature from day of inoculation.

§ Monkey C was control for the examination of the sera of Monkeys M, N, O, P, Q, R. The other tests were made with the controls shown in Table V.

|| Observation period only 20 days (Monkey M) or 21 days (Monkey Q).

had been protected against death by injections of African immune serum or which had received virus attenuated by some method of preservation or by long storage. Nine animals immunized against African virus were available for testing. Particulars with regard to the tests are shown in Table II.

Eight of the monkeys tested had been given a second injection of the African virus from 20 to 48 days after the first as a test of immunity to that virus. The monkeys were tested by inoculation with American virus (F.W. strain) after an interval of between 31 and 39 days from the last injection of African virus, except in one instance in which the interval was 143 days. They were kept under observation for 30 days or more, except when otherwise stated in the table.

Blood was taken from one of the animals before the test inoculation and the serum was tested for protective power against American virus. As the monkey receiving this serum with the virus had irregular fevers not due to the yellow fever virus, it was impossible to know whether an attack of fever was caused by the virus and no conclusion could be drawn, as death does not ordinarily result from the F.W. virus in this laboratory.

All nine of the monkeys immunized against African yellow fever were free from fever following inoculation with the F.W. strain of American virus.

In the cross immunity tests, monkeys immunized against American yellow fever virus resisted African yellow fever virus and, conversely, those immunized with the African virus resisted the American virus.

*Protective Power of American Yellow Fever Sera against African Virus  
and against Leptospirae*

*A. Sera Taken Soon after Undoubted Yellow Fever*

An exceptional opportunity to investigate the relationship of American yellow fever to African yellow fever and to leptospiral jaundice was presented by an epidemic of yellow fever in Rio de Janeiro. Through the courtesy of Dr. H. de B. Aragão and Dr. F. L. Soper we received 15 specimens of serum from 14 persons who had recovered recently from yellow fever in that city. They are listed in Table III under the heading "Recent epidemic," and our findings with regard to these sera are summarized there.

In all the 14 cases a definite diagnosis of yellow fever had been made on clinical grounds. In one case (N.M.) the diagnosis had been proven, for Aragão (15) had transferred the infection to a monkey by means of mosquitoes. In eight cases the attack was described as severe; in six as mild.

Six of the sera were divided in New York and a portion of each was sent to the



TABLE III

*Protection by Sera of Persons Recovered from American Yellow Fever against African Yellow Fever Virus and Leptospira icteroides or L. icterohaemorrhagiae*

Serum	Time after attack	Protection of monkey against yellow fever virus*		Protection of guinea pig against leptospirae*			Experiment	
		Prevented fever	Prevented death	Pfeiffer phenomenon	Prevented fever	Prevented death	Tests with virus	Tests with leptospira
Recent epidemic:								
N. M.	31 days	+	+	-	-	-	I	A
C. F.	39 days	+	+	-	-	+†	I	A
D. A.	26 days	-(1.5)†	-(3.5)†	-	-	-	I	A
M. A.	16 days	+	+	-	-		I	A
(1st specimen)								
F. C.	10 days	+	+	-	-	-	I	A
J. R. S.	85 days	+	+	-	-	-	I	A
G. V.	15 days	-¶	+	-	-	-	II	A
M. R.	49 days	+	+	-	-	-	II	B
M. A.	40 days	+	+	-	-	-	II	B
(2nd specimen)								
J. M.	20 days	+	+	-	-	+†	II	B
M. M.	144 days	-(3.5)§	+	-	-	-	III	C
M. J. S.	153 days	-(2.0)	-(4.5)	+	+	+	III	C
R. B. S.	156 days	**	-(4.0)	-	-	-	III	C
J. M.	153 days	-(4.0)§	+	-	††	††	III	C
C. W. G.	47 days	-(2.0)	-(4.5)	+	+	+	IV	C

\* The yellow fever virus was of the strains Asibi in Experiments I and IV and French in Experiments II and III. The strains of leptospira used were *Leptospira icteroides*, Brazil 49, in Experiments A and B, and *Leptospira icterohaemorrhagiae*, Rat I, in Experiment C.

† The figure in parenthesis is the number of days from inoculation to the first observation of fever in column "Prevented Fever," and from inoculation to death in column "Prevented Death."

‡ Guinea pig had fever but survived and was found to be immune to a second injection of *L. icteroides*.

§ Temperature rose as high as 40°C. only once.

|| Guinea pig had fever, but died on second day after inoculation while being bled; too early for characteristic lesions.

¶ Had unexplained occasional single elevations of temperature from the 1st to the 58th day after inoculation.

\*\* No fever observed.

†† Guinea pig died from unknown cause on day following inoculation.

TABLE III—*Concluded*

Serum	Time after attack	Protection of monkey against yellow fever virus*		Protection of guinea pig against leptospirae*			Experiment	
		Prevented fever	Prevented death	Pfeiffer phenomenon	Prevented fever	Prevented death	Tests with virus	Tests with leptospira
Doubtful cases:								
Car. F.	brief	— (1.5)	— (4.0)	—	**	—	III	C
M. D.	brief	— (1.5)	— (4.5)	—	—	—	III	C
H. D.	brief	+	+	—	**	—	III	C
Dj. A.	brief	+	+	††			III	
Former epidemics:								
G. E.	9 years	— (5.0)	+	—	—	—	II	B
C. H. H.	5 years	— (2.0)	— (5.0)	—	—	—	II	B
D. O. L. (1st test)	23 years	— (2.0)	— (9.0)	—	—	—	II	B
D. O. L., double amount		— (4.0)	+				IV	
I. J. K., 0.4 of amount	8½ years	+	+	††			III	
P. S. R., double amount	30 years	— (2.0)	— (4.5)	††			IV	

†† No serum remained for test.

West African Yellow Fever Commission of the Rockefeller Foundation in Lagos. The results of the tests made by the Commission in Africa were reported by Hudson, Philip, and Davis (21). They found that the sera of N.M., C.F., M.R., M.A., and J.M. protected monkeys against injection of African yellow fever virus (Cases 16, 15, 12, 14, and 17 in their series). One of the sera, however, that of D.A. (Case 13 in their series) failed to protect either of two monkeys against the injection of the virus. Four of these sera, those of N.M., C.F., M.A., and J.M., were tested in guinea pigs against *L. icterohaemorrhagiae*, and in every case the Pfeiffer phenomenon was absent and the animal died of leptospirosis. The results of the protection tests in monkeys were in agreement with those of the tests we performed independently in New York with the same sera, and their Pfeiffer tests gave results consistent with those we obtained using different strains of leptospira.

*Explanation of Tables III and IV.* The results of tests with the sera of persons who had recovered from American yellow fever for protective power against African yellow fever virus and against *L. icteroides* or *L. icterohaemorrhagiae* are shown in Tables III and IV. The results of the tests of the sera themselves are in Table III and those of the corresponding control tests are in Table IV. The tests for protection against yellow fever virus were made in four separate experiments

TABLE IV  
Control Tests in the Experiments Recorded in Table III

Serum	Time after attack	Protection of monkey against yellow fever virus		Protection of guinea pig against leptospira			Experiment	
		Prevented fever	Prevented death	Pfeiffer phenomenon	Prevented fever	Prevented death	Tests with virus	Tests with leptospira
Recovered African native, K. S.	10 mos.	+	+	-	-	-	I	B
Recovered African native, K. Owe.	9 mos.	-(6.5)	+	-	-	+*	II	B
Recovered African native, K. Ot.	22 mos.			-	†	-		C
Monkey A, immunized to African virus		+	+	-	-	-	II	B
Normal <i>rhesus</i> monkey, double amount		-(2.5)**	-(4.5)	-	-	-	IV	B
Anti-icteroides, double amount		-(2.5)	-(6.5)	+	‡	‡	I	A
Anti-icteroides, 1:10 dilution				+	+	+		C
Anti-icteroides, 1:100 dilution				+	§	§		B
Normal horse				-	-	+*		A
Normal human, double amount		-(1.5)	-(4.5)	-	-	-	I	A
Normal human, double amount		-(1.5)	-(4.0)	-	-	-	II	C
Normal human, double amount		-(1.5)	-(3.5)				III	
Normal human, double amount			-(6.0)				IV	

\* Guinea pig had definite febrile attack but survived and was found to be immune to a second injection of *L. icteroides*.

† No fever observed. Death from leptospiral jaundice 5 days after inoculation.

\*\* Temperature rose only to 39.9°C.

‡ Guinea pig had no fever for 17 days, then developed fever and died 6 days later of pneumonia and peritonitis, but without lesions suggesting leptospiral jaundice.

§ Guinea pig had fever and died of peritonitis on the seventh day. No lesions suggestive of leptospiral jaundice.

|| No fever was observed.

TABLE IV—*Concluded*

Serum	Time after attack	Protection of monkey against yellow fever virus		Protection of guinea pig against leptospira			Experiment	
		Prevented fever	Prevented death	Pfeiffer phenomenon	Prevented fever	Prevented death	Tests with virus	Tests with leptospira
No serum, full amount of virus		—(1.5)	—(5.0)				I	
No serum, 0.1 amount of virus		—(2.0)	—(4.5)				I	
No serum, 0.01 amount of virus		—(10.0)	—(11.0)				I	
No serum, full amount of virus		—(6.0)	+¶				II	
No serum, 0.1 amount of virus		—(2.0)	—(4.0)				II	
No serum, 0.01 amount of virus			—(8.5)				II	
No serum, 0.1 amount of virus		—(3.0)**	—(5.0)				III	
No serum, full amount of virus		—(2.5)††	—(4.5)				IV	
No serum, 0.01 amount of virus		—(2.5)	—(4.0)				IV	
Salt solution				—	—	—		A
Salt solution				—	—	—		B
Salt solution				—		††		C

¶ Monkey survived, although it had received no serum.

†† Temperature rose only to 39.8°C.

‡‡ Guinea pig died on night following inoculation.

designated by the Roman numerals I, II, III, and IV, and the tests for protection against leptospirae were made in three experiments, A, B, and C. In order to show which controls belong to each experiment the numbers and letters identifying them are given in the column headed "Experiment."

The sera tested were injected intraperitoneally into *M. rhesus* monkeys in amounts of 1.5 cc. per kilogram of body weight except where otherwise stated in the column headed "Serum." The expression "double amount" means that 3.0 cc. per kilogram was given, and "0.4 amount" means 0.6 cc. per kilogram. These variations in amount of serum do not apply to the tests with leptospirae. The "time after attack" was measured from the onset of the disease to the time of bleeding to procure the serum.

Inoculations of monkeys with African yellow fever virus were given subcutaneously six hours after the serum had been given intraperitoneally. The amount of the virus injected was 0.3 cc. per kilogram of body weight in experiments I, II, and III, and 0.2 cc. per kilogram in Experiment IV.

The temperatures of the monkeys were taken twice each day. To eliminate the personal equation as far as possible, only temperatures of 40°C. or above were considered as signifying fever except when otherwise stated in the tables. In the column headed "Prevented fever" the plus sign (+) signifies that the monkey had no fever and remained well during an observation period of 30 days after inoculation. The minus sign (—) in this column indicates that fever developed, and was probably due to yellow fever, unless explained in a footnote. In the column "Prevented death" a plus sign (+) shows that the animal did not die. Survival was not necessarily due to the action of the serum, for very rarely recoveries follow infection with the strains of African virus in use, as for example in the case of one of the controls receiving no serum. A minus sign (—) in this column signifies that the monkey died of yellow fever. The diagnosis in all fatal cases of yellow fever was determined by finding characteristic lesions on post-mortem examination and on histological examination of the tissues.

In the tests of sera for their power to protect guinea pigs against leptospirae a strain of *L. icteroides*, Brazil 49, was used in Experiments A and B, and one of *L. icterohaemorrhagiae*, Rat I, in Experiment C. Both these strains were given us by H. R. Muller, of The Rockefeller Institute for Medical Research. Strain Rat I had been isolated recently from a wild rat of New York.

In the Pfeiffer tests 1 cc. of the serum to be tested was mixed with 1 cc. of an active culture of leptospirae, and 1 cc. of the mixture was injected immediately into the peritoneal cavity of a guinea pig. Fluid was withdrawn from the peritoneal cavity of the guinea pig 30, 60, 90, and 120 minutes after inoculation and examined under the dark-field microscope. If the Pfeiffer phenomenon was absent, the result was recorded with a minus sign (—) under "Pfeiffer phenomenon." In the cases in which the record shows a plus sign the Pfeiffer phenomenon was present and unmistakable.

The guinea pigs which had been inoculated in the Pfeiffer tests were kept under observation to determine whether they were protected against experimental leptospiral jaundice by the sera injected. In the column "Prevented fever" a plus sign means that the guinea pig remained free from fever and a minus sign that it developed fever. In the column "Prevented death" a plus sign indicates survival, not always due to the serum, and a minus sign means death from experimental leptospiral jaundice. Footnotes explain the irregular results. The diagnosis of leptospiral jaundice in the guinea pig was made by the observation of the jaundice, extreme hemorrhages, and other characteristic lesions, and usually by the observation of leptospirae in the tissues or body fluids.

The tests of the sera of the fourteen persons who had recovered recently from yellow fever in the epidemic in Rio de Janeiro gave the following results:

- 10 persons—the serum protected against African yellow fever virus, but not against leptospirae (tests with *L. icteroides* in 8 instances and *L. icterohaemorrhagiae* in 2).
- 2 persons—the serum protected against leptospirae but not against African yellow fever virus (both tests with *L. icterohaemorrhagiae*).
- 2 persons—the serum protected against neither African yellow fever virus nor leptospirae (tests with *L. icteroides* in one instance and *L. icterohaemorrhagiae* in the other).

The results with the second and third groups of the sera in the above classification were clear cut and regular. Those with the first group showed a few irregularities in the tests in guinea pigs, as is explained in the notes accompanying Table III.

In two cases, in which the guinea pigs died too soon for diagnosis, the definitely negative Pfeiffer test may be accepted as indirect evidence of the lack of protective power in the serum, since in the other cases negative Pfeiffer tests were followed by failure to protect and positive Pfeiffer tests by protection of the guinea pigs. Two guinea pigs inoculated with serum and leptospirae developed fever and recovered. That occasional recoveries are to be expected after inoculation with the strain of *L. icteroides* used, in the absence of a protective serum, is shown by the behavior of a control guinea pig which received the same strain with normal horse serum and recovered (Table IV).

The two species of leptospirae, *L. icteroides* and *L. icterohaemorrhagiae*, appear to be so closely related that they may be used interchangeably in immunological tests for leptospiral jaundice, as in this investigation of ours, and we have already referred to the work of investigators who obtained strong evidence of their serological identity. It will be noticed in Table IV that anti-icteroides serum, even when diluted, gave positive Pfeiffer reactions with both species of leptospira. This serum had been prepared by The Rockefeller Institute by immunizing a horse against a number of strains of *L. icteroides*.

The absence of protective power against African yellow fever virus in four of the sera from recent cases of yellow fever in Rio de Janeiro (D.A., M.J.S., R.B.S., and C.W.G.) is in our opinion strong evidence against the presence of yellow fever in these cases, in view of the evidence already presented showing that African yellow fever and American yellow fever are immunologically the same. That some of the

sera sent from Rio de Janeiro should be from cases other than of yellow fever casts no reflection on the ability of the diagnosticians, for in many cases it is impossible to distinguish by symptoms between yellow fever and leptospiral jaundice, and mild yellow fever may simulate a number of infections.

As evidence of the lack of protective power in these sera we have the results following the inoculation of one monkey in each case, and the confirmatory observations, already mentioned, of Hudson, Philip, and Davis (21) in the case of D.A. The evidence from the inoculation of one monkey in each case seemed conclusive, however, as there was no suggestion of even partial protection by amounts of serum varying from 3.2 cc. to 5.9 cc. according to the weights of the animals.

Observations which bear on the reliability of such negative protection tests have been tabulated by Hudson (22) and relate to sera of 23 West African natives who had recovered recently from yellow fever during observed epidemics. Of 28 monkeys, each of which had received 4 cc. of serum and 0.25 cc. to 1.0 cc. of blood virus (Asibi strain), only one succumbed to yellow fever. The possibility of error of diagnosis in the case of the person supplying the one negative serum cannot be completely ruled out. Hudson gives the estimated number of spontaneous recoveries of *rhesus* monkeys after inoculation with the Asibi strain of virus as about two per cent.

Two of the sera from Rio de Janeiro (M.J.S. and C.W.G.) had strong protective power against *L. icterohaemorrhagiae*, and in one of these cases (C.W.G.) the occurrence of a definite relapse after discharge from the hospital is against the diagnosis of yellow fever and suggestive of leptospiral jaundice, which so commonly has an after-fever. H. R. Carter (23), after a wide experience with yellow fever, said of that disease, "I have never seen a relapse, but other men have. They must be rare." Although the possibility cannot be entirely excluded that the protective substances in either serum were due to an earlier attack of leptospiral jaundice, it is highly probable that in both cases the illnesses taken for yellow fever were in reality leptospiral jaundice (Weil's disease).

M.J.S. had a mild attack and was discharged from the hospital as cured on the seventh day after the onset, according to data published by Barreto (24).

C.W.G. came to New York after his recovery and permitted us to draw an additional specimen of blood. He also supplemented the information which had been sent us by Dr. F. L. Soper. The illness of C.W.G. was characterized by high fever, headache, great weakness, vomiting, backache beginning with the

second day, moderate albuminuria from the fourth day, and jaundice from the seventh. During the first four days the symptoms were such that the physicians were of the opinion that the disease was not yellow fever, but on the seventh day a definite diagnosis of yellow fever was made. The patient was allowed to get up on the 13th day and to leave the hospital on the 15th. On reaching home he again developed high fever and headache. On the 17th day he was able to leave his bed. Convalescence was slow, and he was still distinctly jaundiced on the 48th day, when he visited our laboratory in New York. In Rio de Janeiro he worked at the water front and docks in places where there were many rats. An investigation of this case in Brazil has been published by R. A. Warner (25).

As the sera from most of the 14 persons in Brazil who had recently had symptoms like those of yellow fever possessed strong protective power against African yellow fever virus, the conclusion seems justified that the yellow fever of America is the same disease as that of Africa. The same conclusion has been reached by Theiler and Sellards (26), Hudson, Bauer, and Philip (27) and Hudson, Philip, and Davis (21) as the result of their protection tests with American sera.

### *B. Sera Taken Soon after Suspected Yellow Fever*

Four of the sera sent us came from a town in southern Brazil, and were from cases in which yellow fever was only suspected. In each case the illness was mild and a diagnosis could not be made from the symptoms. These specimens were tested with the results recorded under the heading "Doubtful cases" in Table III. Two of the sera protected completely against yellow fever and two did not protect at all. One of the sera which protected against the virus and the two others were tested for power to protect against *L. icteroides*, and none protected. We were evidently dealing with two cases of yellow fever, and two cases of infection other than yellow fever or leptospiral jaundice.

### *C. Sera Taken Long after Yellow Fever*

The testing of the sera from recent cases of yellow fever at Rio de Janeiro indicated that the Brazilian disease was the same as that of Africa. The assumption was also that it was identical with the disease studied by Reed in Cuba and suppressed by Gorgas in Panama. The fact, however, that the ocean passage between Senegal and Brazil may be as short as six days permits of the possibility of the transport of the African yellow fever to Brazil, although there is no evidence of this at the present time. Fortunately the relationship of the present yellow fever of Africa and Brazil to the historic yellow fever of America



need not be left to speculation. Direct evidence has been obtained by the examination of the sera of persons who passed through attacks of American yellow fever many years ago.

Serological evidence of the unity of the American yellow fever of the past and the African yellow fever of the present has been secured by Bauer and Hudson (28). Two sera obtained 23 years after attacks of yellow fever in the Panama Canal Zone and one taken 26 years after an attack in Tampico, Mexico, protected monkeys against African yellow fever. A fourth serum taken 26 years after yellow fever in New Orleans failed to protect. The tests were made in duplicate.

The results of the tests of five sera from persons who had had yellow fever in the Americas many years ago are given under the heading "Former epidemics" in Table III. The particulars regarding the sources of these sera are as follows:

G.E. had yellow fever in La Union, Salvador, in July, 1919, soon after arriving from Honduras. The attack was very severe and lasted 14 days. It was characterized by much vomiting, heavy albuminuria, and jaundice. According to the patient there were other cases of yellow fever in La Union and in Honduras at the time. Blood was obtained for us by Dr. J. E. Elmendorf, Jr., and information regarding the case was supplied by Dr. Peralta Lagos. The interval between the attack and the bleeding was 9 years and 3 months.

C.H.H. had yellow fever in Aracajú in the State of Sergipe, Brazil, in May, 1923, according to his statement. We are indebted to Dr. B. E. Washburn and Dr. Hargreaves of Kingston, Jamaica, for obtaining blood. The blood was drawn 5 years after the attack.

D.O.L. had a very severe attack of yellow fever in the city of Panama in June 1905. He was attended by several doctors who had had a large experience with yellow fever, including Colonel W. C. Gorgas, and Dr. H. R. Carter. We are indebted to Dr. W. E. Deeks and Dr. R. C. Connor for putting us in touch with D.O.L., and to Captain J. W. Smith of the Medical Corps of the U. S. Army for drawing a specimen of his blood. The interval between the attack of yellow fever and the drawing of blood was 23 years and 5 months.

I.J.K. contracted yellow fever in Morropón, Peru, in April, 1920, during an epidemic, and had a mild attack. He spent a few months in West Africa in 1926. In his case the interval between the disease and the bleeding was 8 years and 7 months. A different specimen of serum of I.J.K. was tested by Hudson, Bauer, and Philip (27) and found to protect against African virus, Asibi strain.

P.S.R. had yellow fever in Havana, Cuba, in May, 1899, 30 years before the specimen of serum was taken.

The results obtained in these tests were as follows:

Serum taken 8½ years after an attack of yellow fever in Peru protected a monkey completely against African yellow fever. Serum taken 9 years after yellow fever in Salvador permitted the appearance of fever in the monkey but prevented death. A specimen obtained 23 years after an undoubted attack of yellow fever in Panama failed to protect when injected in the usual amount, but prevented death, while permitting fever, when used in double quantity. The same amount of normal human serum failed to prevent the death of a control animal in this experiment and in each of three other experiments (Table IV). A serum taken five years after yellow fever in Northern Brazil failed to protect. The evidence with regard to the diagnosis in this case was meager. Serum taken 30 years after yellow fever in Cuba did not protect.

The results obtained in these tests and those of the other investigators cited show the historic yellow fever of America to have been the same as the present African yellow fever.

#### *Protective Power of African Yellow Fever Sera against American Virus*

If we were right in our conclusion, derived from observation of the protective power of American sera against African virus, that the yellow fever of America is the same as that of Africa, then the sera of persons who have recovered from yellow fever in Africa should protect likewise against American yellow fever virus.

To demonstrate this by experiment proved difficult owing to the low virulence of the F.W. strain of American yellow fever virus for monkeys, as the strain exists in this laboratory. Only twice in our experience has death resulted from the inoculation of a monkey with the F.W. strain. In using this strain in protection tests it was necessary, therefore, to base our conclusions on the presence or absence of fever in the experimental animals after inoculation. Under these circumstances the results of the tests would of necessity be inconclusive in relation to the individual specimen of serum, but they should permit conclusions with regard to a group of sera when compared with an adequate series of control tests.

In Table V are shown the results of tests of the sera of six natives of the Gold Coast of West Africa. All of these persons had had yellow fever in observed yellow fever epidemics. We are indebted to Dr. Henry Beeuwkes, Director of the West African Yellow Fever Commission of the Rockefeller Foundation, for these sera.

TABLE V

*Protection by Sera of Persons Recovered from African Yellow Fever against American Yellow Fever Virus*

Serum	Time from attack to bleeding	Protected monkey from fever after inoculation with American yellow fever virus, F.W. strain*	Result of later test inoculation of monkey with African virus, Asibi strain	Results of other tests of the sera
African natives.				
K. Ot.	22 mos.	+	No reaction	Did not protect against <i>L. icteroides</i> (Table IV)
K. B.	23 "	+	No reaction	Protected against African yellow fever virus (Asibi)†
K. Owir.	20 "	+	Survived‡	
J. O.	19 "	+	No reaction	
K. N.	30 "	+	No reaction	
T. C.	33 "	+§		
Controls:				
American, recent recovery, M. A.	40 days	+	No reaction	Protected against African yellow fever virus (Table III)
American, recent recovery, C. W. G., double amount	47 "	-(11)∥	Survived‡	Protected against <i>L. icterohemorrhagiae</i> , but not African yellow fever virus (Table III)
Anti-icteroides, double amount		-(3.5)∥	No reaction	Protected against <i>L. icteroides</i> and <i>L. icterohemorrhagiae</i> , but not African yellow fever virus (Table IV)
Normal human, double amount		-(4.0)∥	No reaction	Similar specimens in Table IV

\* The F. W. strain showed low virulence for monkeys and did not cause any deaths during this experiment.

† Tested in a later experiment with controls. Monkey showed fever but was protected against death.

‡ Had brief fever reaching 40°C. but recovered.

§ Had no fever but died on 25th day of observation from cause other than yellow fever.

∥ Fever indefinite or absent. The highest temperature of the monkey in the case of C. W. G. was 39.8°C.; in the case of the anti-icteroides serum, 39.7°C.; in the case of the normal human serum, 39.9°C.; in the case of the small dose of virus, 39.8°C.

TABLE V—*Concluded*

Serum	Time from attack to bleeding	Protected monkey from fever after inoculation with American yellow fever virus, F.W. strain*	Result of later test inoculation of monkey with African virus Asibi strain	Results of other tests of the sera
Controls— <i>Continued</i> : Normal <i>rhesus</i> monkey, double amount		— (4.0)	No reaction	Similar specimens in Table IV
No serum, full amount of virus		— (4.0) ¶		
No serum, 0.01 amount of virus		— (3.5)	No reaction	

¶ Died from dysentery on 28th day after inoculation.

The methods of performing these tests and tabulating the results are in general the same as in the previous experiments (Tables III and IV). The regular amount of serum injected into the monkeys was 1.5 cc. per kilogram of body weight, and the double amount was 3.0 cc. The amount of virus-bearing blood injected was uniformly 0.4 cc. per kilogram, and it was injected subcutaneously six hours after the serum had been given intraperitoneally.

The results of the tests, though clearly unreliable in the individual case on account of the low virulence of the F.W. strain of virus used, show that the African sera as a group possessed protective power against American yellow fever virus. In no case did fever appear in a monkey which had received one of these six sera. The three control animals which received the full amount of virus without serum or after normal human or monkey serum all showed rises of temperature to 39.9°C. or over at the time when fever would be expected as the result of the inoculation. These observations are in agreement with the stronger evidence obtained by testing American sera against African virus.

*Differences between the American and African Strains of Yellow Fever in Their Virulence for Monkeys*

The two African strains we used in our experiments (French and Asibi) were similar to each other in virulence for monkeys, but they

differed markedly in this respect from the one American strain (F.W.). To facilitate comparison, we have summarized our experience with the inoculation of *rhesus* monkeys with the Asibi and F.W. strains.

In doing so we have considered only those cases in which the inoculum was blood taken on the first day of fever from monkeys experimentally infected with yellow fever in this laboratory. The amount of blood was not less than 0.1 cc., expressed as undiluted whole blood. If citrated blood was injected, the specimen was not over 24 hours old, and, if dried blood was used, it was prepared by drying in vacuum in the frozen state and was not over 100 days old. In fatal cases the animals were allowed to die or were killed when moribund.

Of 24 monkeys inoculated with African virus of the Asibi strain, all came down with yellow fever and only one survived. Of 20 monkeys receiving American virus of the F.W. strain, 15 developed fever and two of these died. These two, Monkeys V and W, had received dried blood 94 and 63 days old, respectively.

In the cases of those animals that showed temperatures of at least 40°C., the incubation periods for the Asibi strain were, minimum 1.5 days, maximum 6, and average 2.7; for the F.W. strain, minimum 1.5 days, maximum 12, and average 4.3. The intervals between inoculation and death were, for the animals inoculated with the Asibi strain, minimum 2.5 days, maximum 10.5, and average 5. The two animals which died after receiving the F.W. strain did so 4.5 and 8 days after inoculation.

The Asibi strain of African virus proved to have a much higher virulence for monkeys than the F.W. strain of American virus. The former caused death in 23 of 24 monkeys inoculated, and the latter in only 2 of 20. It does not follow, however that there is a similar difference between these strains in their virulence for man, nor that these strains are representative in virulence of the African and American strains in general. Dr. N. Paul Hudson tells us that the figures of the laboratory of the West African Yellow Fever Commission of the Rockefeller Foundation showed, up to the end of 1928, that there was a marked variation in the virulence of African strains for *Macacus rhesus*. A strain (H.P.) obtained from a fatal case in a European killed only one-third of the monkeys inoculated with blood drawn from infected monkeys at the time of fever. The mortality was low also when this strain was transmitted from animal to animal by mosquitoes.

*Comparison of the Lesions Produced by African and American Strains of Yellow Fever Virus*

The gross and microscopic lesions produced by the Asibi and French strains of African yellow fever virus in our monkeys were in general those described by Hudson (29) for experimental yellow fever as produced in *M. rhesus* by the Asibi strain. The two animals (Monkeys V and W) which died as the result of inoculation with the F.W. strain of American virus showed gross and microscopic lesions such as are produced by the African strains. One animal (Monkey X) killed during a mild attack following inoculation with the F.W. strain showed only very slight changes.

Da Cunha and Muniz (30) speak of the varying extent of the liver lesions in monkeys after inoculation with a Brazilian strain of yellow fever virus. In some animals they found extensive necrosis of the hepatic cells and none in others. Although they found that their virus possessed less virulence than an African strain, it was sufficient at times to cause early death with extensive lesions like those following inoculation with the African strain.

#### CONCLUSIONS

1. The yellow fever now in South America, the present yellow fever of Africa and the historic yellow fever of Panama and other American countries are the same disease. This conclusion is based on cross immunity tests in monkeys with strains of yellow fever virus from Africa and Brazil and on tests of sera from 25 persons, who had recovered from yellow fever in various places and at various times, for the power to protect monkeys against African or Brazilian virus strains.

2. Cases of leptospiral jaundice (Weil's disease) were present among those diagnosed as yellow fever in the recent epidemic in Rio de Janeiro. This is shown by the isolation of cultures of leptospirae from the blood of two patients by H. R. Muller and E. B. Tilden of The Rockefeller Institute, and by the demonstration by us of protective power against leptospirae and absence of protective power against yellow fever virus in the sera from two persons after recovery. The isolation of leptospirae by Noguchi and other investigators from the

blood of occasional patients in past epidemics of yellow fever in a number of American countries indicates that leptospiral jaundice was present then as well and was diagnosed clinically as yellow fever.

3. The absence of protective power against leptospirae shown by the Brazilian sera which protected against yellow fever virus and the absence of protective power against yellow fever virus in the sera that protected against leptospirae point to the probability that American yellow fever is not the combined effect of leptospirae and yellow fever virus. The position of *L. icteroides*, isolated by Noguchi during yellow fever epidemics, now appears to be not that of a secondary invading microorganism in cases of virus yellow fever, but that of the incitant of a form of infectious jaundice, sometimes fatal, often coincident in its appearance with typical yellow fever and apparently indistinguishable from it clinically. This leptospiral disease has not hitherto been separated from true yellow fever. Noguchi's discoveries become, therefore, of the greatest significance in respect to the epidemiology and causation of yellow fever and of infectious jaundice, previously confused one with the other. In all outbreaks of supposed yellow fever hereafter the existence of the two kinds of jaundice, one due to yellow fever virus and the other to leptospirae will have to be taken into account. Only the former probably is spread by mosquitoes and requires anti-mosquito measures for its control.

4. The only difference observed by us between the American and African strains of yellow fever virus was a pronounced difference in virulence for monkeys. The virulence of the two African strains studied was very high while that of the one American strain was highly variable and usually low.

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# THE IMMUNOLOGICAL SIGNIFICANCE OF COLOSTRUM

## III. INTRANUCLEAR BODIES IN RENAL DISEASE OF CALVES

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PLATES 9 AND 10

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The urinary organs of young calves are vulnerable, and pathological conditions readily appear in an abnormal environment. Of these the so-called spotted kidney has been discussed in an earlier paper.<sup>1</sup> In this condition evidence points to a sublethal infection with *B. coli*. The process involves both organs alike. The multiple white foci of infiltrated cells are situated in the cortex and are embedded in normal tissue. The process ends either in a reabsorption of the exudate cells or in sclerosis whereby the enclosed functional structures are destroyed. The urinary secretion contains only a trace of protein and the lesion is not recognized until the animal is killed. The kidneys are as a rule somewhat heavier than the normal organ.

Another pathological condition expresses itself in large, uniformly white kidneys symmetrically involved. The lesions of the spotted kidney may be present also. Cases of the white kidney were observed several times in calves reared in the usual way about 10 years ago. Latterly experiments involving the withholding of colostrum and the feeding of serum in its place have brought a few to the surface. Before discussing the probable nature and etiology of the condition a few cases will be presented. To begin with, a case will be described in which the presence of intranuclear bodies was associated with nearly normal organs.

*Calf 1532.*<sup>2</sup> Holstein male born Jan. 11, 1929. Received in 3 consecutive doses, up to the 13th hour, 1400 cc. of cow serum mixed with cow's milk. 700 cc. of the

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<sup>1</sup> Smith, T., *J. Exp. Med.*, 1925, 41, 413.

<sup>2</sup> This calf is tabulated among others in *J. Exp. Med.*, 1930, 51, 488.

serum came from untreated cows and 700 cc. from a cow treated with *B. coli* filtrates (D) and found only feebly protective for guinea pigs.<sup>3</sup> The serum had been stored for nearly 2 years. No significant amount of specific protective antibodies had thus been fed to this animal.

During the 2 months of life there were no disturbances except a brief period of liquid stools during the 2nd day. The urine following the heat test became faintly clouded throughout but there was no measurable deposit<sup>4</sup> of coagulum except on one occasion when it was 1.3 per cent. The gain in weight was normal, also the temperature curve. It was killed by a blow and severing neck vessels when 2 months and 8 days old. The following represent the few deviations from the normal found at autopsy.

The lymphoid tissue of the small intestine was below the normal in quantity but the thymus was normal in weight (300 grams). In the upper portion of the duodenum the mucosa was dotted with roundish elevations which were later recognized in sections as a lymphocyte infiltration of the villi. In the cecum the veins of the submucosa were marked by pigment deposits. The same condition extended 15 or 17 cm. below the ileocecal valve. The free border of the valve was deeply pigmented. In the upper colon were two areas showing numerous vague whitish spots 1-2 mm. in diameter. In sections, these spots were shown to be lymphoid follicles in submucosa. They were pear-shaped and the tapering portion extended into the mucosa between the tubules. A small number had reached the surface in the form of cylindrical plugs. Over them the surface epithelium had disappeared.

The kidneys were about 1½ times the average weight at this age (192 and 200 grams, respectively, with capsule stripped off). On section, faint pale radial striations could be detected in cortex. In frozen sections of the fresh tissue, the condition shown in Fig. 1 presented itself. In the columns of Ferrein the epithelium of the tubules contained irregular foreign bodies all located within the nuclei. Some were in cells of the convoluted tubules. They appeared as irregular agglomerations of 2μ discs, some 4 to 6 consolidated into a straight or bent line. The epithelium was not distorted or distended as yet. In scrapings of the fresh (unfrozen) tissue, the bodies were present, hence freezing had nothing to do with bringing them out. In sections of tissue fixed in Zenker and alcohol, they were not changed and were colored reddish in stains containing eosin. In general the kidney tissue appeared normal, with perhaps a very slight hyperplasia of the interstitial tissue. In Figs. 2 and 3 a thin film of scrapings of the cut surface stained in hematoxylin and eosin brings out the intranuclear position of these bodies. The cytoplasm of the epithelial cells has been broken up by the procedure and only the nuclei have retained their form.

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<sup>3</sup> For the method of testing protective power see *J. Exp. Med.*, 1930, 51, 474. For a brief description of the cows furnishing serum see *J. Exp. Med.*, 1930, 51, 483.

<sup>4</sup> For the rough estimation of protein coagulum in urine see *J. Exp. Med.*, 1930, 51, 485.

This is the first and thus far the only animal in which the intraglobular bodies were found in mass under apparently still normal functioning of the kidneys and normal condition of the animal generally.

The following fatal case is perhaps typical of the uncomplicated syndrome. Death took place during the writer's absence and only the formalinized kidneys and Zenker-fixed portions were available.

*Calf 1511.*—Guernsey bull, born May 22, 1928. Fed 700 cc. serum of Cow C<sup>5</sup> (treated with *B. coli* mutant 1192<sub>b</sub>) mixed with milk, in 3 doses as heretofore, the last dose when calf was about 13 hours old; thereafter only milk.

There were no disturbances of noteworthy extent during the 1st month. It was turned out into a paddock at this time and fed alfalfa in addition to the milk diet since it refused hay and grain. During the 2nd month it became unthrifty and constipated, the coat rough and abdomen distended. July 19, the calf was found down. It took milk very slowly. There was present tenesmus with passage of small amounts of feces. On July 20, it took a little milk early and was found dead at noon. The temperature of this calf taken twice daily was within normal range throughout if we except a slight rise on the 4th, the 27th–31st, and the 46th day.

*Autopsy.*<sup>6</sup> Mucous membranes pale. Subcutaneous tissues around buttocks and scrotum edematous, the fluid, blood-tinged. Both kidneys enclosed in a layer of edematous tissue. The left is also enclosed in a blood clot. The bladder and rectum are surrounded by edematous, blood-tinged tissue. On dorso-ventral tip of spleen a projecting, flattish, hemorrhagic elevation. When the kidneys are shelled out of the edematous envelopes, they are found larger than normal. The weights are for the left kidney 540 grams, for the right 460 grams. This is between 2 and 3 times the weight of normal kidneys at this age. Both appear uniformly white with numerous hemorrhagic points near surface from  $\frac{1}{2}$  to 2 mm. apart. On section, cortex whitish throughout with broad injected vessels radiating from the medullary boundary into both cortex and medulla.

The progress of the renal disease was reflected to a certain extent in the urine. Up to and including the 39th day of life there was a faint clouding when it was acidified and boiled. No further samples were tested until the day before death when the coagulum formed after heating was equivalent to 22.6 per cent in volume after 24 hours' sedimentation. Urine taken from bladder at the autopsy had the same volume of coagulum.

The kidneys hardened in Zenker's fluid and formalin were available for further study. Transections of the entire organ indicated some interference with the outflow since the calices were separated by a distinct space from the papillae. Stasis was further indicated by a general distention of the convoluted tubules

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<sup>5</sup> See *J. Exp. Med.*, 1930, 51, 484.

<sup>6</sup> By Dr. R. B. Little in the writer's absence.

beginning at or within the glomerulus. The cortical structures were also driven apart by edema. The interstitial connective tissue was slightly increased and this was moreover evidenced by occasional mitoses. Within the nuclei of the epithelium of the convoluted tubules were inclusions, in form disclike, about  $2\mu$  in diameter (Figs. 4 and 5). The number of discs in a nucleus varied up to 12. They were fused together in an irregular, longitudinal mass. As a result some cell nuclei were greatly distended. One measured  $15\mu$  by  $20\mu$ . Any definite geometrical form was not recognizable except the generally circular outline of the discs. The fused masses have sharp borders, homogeneous surface, and suggest crystals. The intranuclear situation is easily recognized except when the masses are largest. Even then the greatly distended nuclear membrane may be made out. They are found in the labyrinth and the columns of Ferrein and quite irregularly in these regions.

The hemorrhages are of two kinds, those starting from glomeruli and those about the loops of Henle. Scattered about are groups of hemin crystals. Hyaline casts are found in the smaller collecting tubules and Henle loops.

In the hardened tissue, in cross sections of some of the distended tubules, there are from one to all of the epithelial cells with intranuclear bodies. The involved cell usually projects well beyond its neighbors into the lumen. Rarely a group of two or three hypertrophied cells shed into the lumen nearly occludes it. In the medulla, occasional greatly dilated collecting tubules are encountered. In others, hyaline casts are present. Associated with these inclusions there is hypertrophy and some degeneration of cells in both primary and secondary convoluted tubules and moderate interstitial edema together with some cell infiltration probably monocytic. The striking feature is the general distension of tubules from Bowman's capsule down.

Some tests with acids and alkalis were made with frozen sections of the formalinized kidneys. Thus 4 per cent NaOH acting upon the sections for 18 hours caused no change in the inclusions. Similarly 5 per cent acetic acid failed to dissolve them, although they seemed a trifle smaller.

The following is another typical case.

*Calf 1411.*—Holstein male, born Jan. 12, 2.40 p.m. Receives 400 cc. serum of Cow D (*B. coli* filtrate) plus an equal quantity of milk. At 8 hours it receives 200 cc. and at 15 hours 100 cc., also with milk added; thereafter only milk. This animal passed through a relatively severe attack of liquid stools which lasted 5 days. Later, traces of protein appeared in the urine from time to time. On March 4, the urine was clouded with fine flocculi which were covered with masses of small diphtheroid rods. Cultures brought to light *B. pyogenes* and the specific cystitis bacillus.<sup>7</sup>

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<sup>7</sup> Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1926, 44, 11.

March 23. Calf tends to lie down. Grits its teeth. Takes only a portion of the offered milk. Temperature about normal.

March 30. Calf has been growing weaker and taking only about one third the normal amount of milk. Marked constipation. Knee joints weak and evidently painful but not swollen. Urine contains a coagulable deposit equal to one third the bulk of entire fluid. Animal killed and autopsied.

The following abnormalities were noted. The rumen contains a very large quantity of hair intimately mixed with the semisolid vegetable contents. The fourth stomach contains a viscid white fluid and some twine from the bedding. Some petechiae in the leafy portion. In the large intestine, feces are dry and formed into hard pellets.

The perirenal fat is edematous, and from  $\frac{1}{2}$  to 1 cm. thick, owing to the accumulation of a clear colorless fluid in its interstices. The capsule easily removed. The cortical tissue is very pale, without normal markings and dotted with dark red points, about 1 mm. or less apart. Under capsule are scattering hemorrhages and clots, several in each lobe and from a few to 15 mm. in diameter. On section, the same dark red points throughout cortex. Medulla without noticeable changes. Kidneys, after removal of fat, weigh each 350 grams, *i.e.* they are nearly twice normal weight.

Free hand and frozen sections of these organs show lesions of the epithelium of the convoluted tubules. Within the nuclear membrane is a group of fused disclike bodies of moderate refringence and slightly brownish tint. The individual discs composing the mass are 1 to  $2\mu$  in diameter. The masses themselves 5 to  $10\mu$  in diameter. Besides these intranuclear bodies there are larger masses of similar material irregular in form gathered in lumina of tubules and blocking them in part or wholly. 5 per cent acetic acid does not disturb them. Normal NaOH acts upon them and after several hours reduces them to heaps of minute granules.

Sections of kidney tissue fixed in Zenker's fluid show a marked irregular dilatation of the convoluted tubules. Nuclear membrane of the epithelial cells irregularly folded, one portion pressed toward the opposite half. They are 2 to 3 times normal size and contain faintly stained foreign masses. The glomeruli are either normal or else contracted and pressed to one side. The glomerular space is filled with a bubbly mass or else with red corpuscles. Hemorrhages are widespread in convoluted tubules, with the glomeruli as the probable source of the blood. The columns of Ferrein appear more compact than is normal, suggesting slight hyperplasia of the stroma. Hyaline casts present. In the collecting tubules hyaline casts numerous.

In certain animals showing renal lesions intranuclear bodies were not detected. However, there were observed casts either in the urine or in the collecting tubules made up of apparently the same material as that within the nuclei of the epithelial cells. Details of these cases are omitted.

An illustration of the association of the lesions of the spotted kidney with the type under discussion is furnished by a calf<sup>8</sup> which received colostrum after a delay of 22 hours. The kidneys of this animal closely resembled those of No. 1511. Intranuclear bodies were present and located chiefly in the labyrinth.

In the course of experiments<sup>9</sup> on the substitution of serum for colostrum a certain number of calves died unexpectedly in the 2nd month of life during the night or quickly after convulsive seizures during the day. In one of the animals thus affected intranuclear bodies were found. The relation between this acute fatal affection and the more slowly developing renal lesions is an open one. There are, however, reasons for tentatively regarding the renal and the convulsive type as allied etiologically. The following case among several is therefore briefly outlined.

*Calf 1416.*—Holstein heifer, weighing at birth 70–75 lbs. It received in place of colostrum during the 1st day 700 cc. of serum of Cow B (treated with heated *B. coli*). Slight proteinuria on 2nd day disappearing on 3rd day. On the 50th day of life, the calf refuses part of the usual amount of milk and develops symptoms due to irritation of central nervous system. The left ear is held back against head. The calf moves at times in a circle with unsteady gait. The left side of head and trunk is pressed against the sides of stall leading to contusions of head on that side. It is evident after a time that the calf fails to see. The pupils are dilated. Later in the day the animal lies down, with head drawn to the left and right ear twitching. Gets up and walks in a circle. Profuse sweating. The next day calf is unable to swallow its food. Gait unsteady. Forelegs spread apart. Knees and ankles bent. The symptoms remained unchanged until the 4th day. The calf began to take milk from a bottle. Blindness continued. During these days the urine contained about 1 to 1.5 per cent coagulable deposit and the temperature did not rise above 39.5°C. The case was tentatively considered one of botulism poisoning. The specific bacillus could not be demonstrated in the fecal discharges. Constipation was marked and the feces were in the form of small balls.

97th day. Calf has remained blind, but has recovered in other respects. Urine has contained from 1 to 3 per cent coagulable material up to the present. Killed today. The abnormal conditions noticed are as follows:—Digestive tract normal except lower half of large intestine which is filled with very dry fecal balls 1 to 2 cm. in diameter. Slight, rather firm adhesions of right cephalic and ventral lobe of lungs to ribs. Collapse of scattering lobules in cephalic lobe. Both kidneys en-

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<sup>8</sup> Smith, T., *J. Exp. Med.*, 1925, 41, 422.

<sup>9</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, 36, 453.

larged and with uniformly pale cortex, increased in thickness. Medulla sharply demarcated from it by fullness of vessels. Weight of each kidney, freed from fat envelope, about 305 grams.<sup>10</sup> Some slight adhesions of caudal border of liver to kidneys and other structures. A trace of fat in liver cells. Malpighian bodies of spleen very distinct. Ovaries cystic. Brain and medulla without visible abnormalities.

In free hand and frozen sections of kidneys, the epithelium of the convoluted tubules contains the same kind of material described under Calf 1411. There are no casts of this material in the lumina.

Examination of fixed material from kidneys shows a slight distension of convoluted and straight tubules in limited areas throughout cortex. Scattering interstitial foci of cells made up of polynuclear and some mononuclear leucocytes. The enveloped tubules filled with polymorphs. Epithelium of convoluted tubules with enlarged and irregularly folded nuclei, appearing like collapsed spheres. In certain areas there are intranuclear discs stained reddish. Casts present but in small numbers. Sections of liver, spleen and brain without noticeable abnormalities.

This is the only animal to survive the acute attack. The renal lesions resembled those of the group in which such an attack was not observed. However, in several acutely fatal cases the highly congested kidneys did not show intranuclear bodies.

#### DISCUSSION

The observations set down in the preceding condensed protocols are published at this time because of the interest manifested in similar structures associated with certain infectious diseases and also because the investigations have been discontinued for the time being. The material is relatively scarce, it has been available at unexpected, inopportune moments, and in certain instances it was undiagnosed during life. It nevertheless contains a certain interest in that it presents several significant problems in renal pathology.

As regards clinical indications it may be said that they were relatively scarce. Proteinuria was present only towards the end of life. The amount of protein was then quite large. Constipation was marked in several animals. The temperature rarely rose more than a degree C. and usually in association with diarrheal conditions.

The intranuclear bodies were observed both in an otherwise still

<sup>10</sup> Normal weight at this age about 200 grams.



normal kidney and in organs markedly altered. The chief characters are enlarged white organs covered with an edematous layer involving the perirenal fat. The cortex is peppered with petechial hemorrhages which on section are seen to be present throughout the cortex. The distension of the entire convoluted tubule system of the labyrinth was present in some, and of only certain portions in others. Isolated collecting tubules were also greatly distended. In the formalin-fixed kidneys, the calices of the pelvis were distended. Inflammatory reactions, such as interstitial focal collections of cells and hyperplasia of stroma, may or may not be present. Hyaline casts are frequently seen, chiefly in Henle's loops. Bacteria are absent both in cultures and in sections of the organ.

The intranuclear bodies are in all cases alike. They are made up of roundish disclike units, 1 or 2 $\mu$  in diameter, fused into masses containing up to a dozen discs. The fused masses are irregular in form and suggest concretions. Weak acids and alkalies have not dissolved or otherwise changed them materially. They are situated mostly in the epithelium of Henle's loops, more rarely in the convoluted tubule epithelium. These bodies are not of common or frequent occurrence in the calf. The kidneys of many calves killed after 2 months and apparently normal were searched in vain for these bodies. A study of the tables in another publication<sup>11</sup> brings out the fact that the renal lesions were found only in calves in which there was a deviation from the normal colostrum feeding, either by substituting serum, or by delaying the intake of colostrum, or allowing only a small amount of colostrum at the start. However, only a small per cent of these were affected. It is significant that none of the 14 controls<sup>11</sup> allowed to feed normally with the dam for 3 or 4 days were abnormal when kept 4 to 10 weeks.

Thus far no information bearing on the nature of the intranuclear bodies is at hand. Their appearance suggests some form of concretion, deposited or formed within the nuclear membrane. The involvement of the cells may be quite extensive, almost universal, as in No. 1532 (Figs. 1-3).

The writer has been unable to find in medical publications references

<sup>11</sup> Smith, T., and Little, R. B., *J. Exp. Med.*, 1930, 51, 483.

to renal conditions similar to the ones described, except a paper by Van Leersum.<sup>12</sup> In studying urinary calculi in rats on a vitamin-A-deficient diet he came to the conclusion that the calculi are not merely deposits of urine salts but made up of "epithelial cells impregnated with calcium." He did not, however, trace the process back towards its beginnings in the attached cell.

The effect on the epithelial cells is at first almost negative. Later the cells become hypertrophied as the inclusions grow and project into the lumen of the tubules. In one case a mitotic cell was detected in which the inclusion had been pushed into the cytoplasm. The cell was thus still capable of reproduction. Whatever the final effect, the degenerative process appears to be slow. The end result, as suggested above, is probably the shedding of the cell and its replacement by mitotic multiplication at least for a time.

Taking the various gross and microscopic data into consideration, we may conclude that the main disturbing factor is occlusive and that occlusion is due to a shedding of the involved epithelium with the formation of casts in the tubules and possibly later on concretions obstructing also the ureter. The perirenal edema and multiple petechial hemorrhages favor this theory. The presence of numerous casts in certain cases not here reported made up of dislike bodies is contributory evidence. It is conceivable that in the animal (No. 1532) in which the epithelium of the still normal kidneys was universally involved, the simultaneous discharge of these concretions might cause blocking of the entire excretory system. Complications of this type of injury, with lesions due to the "spotted kidney" form of infection with *B. coli*, are evident in some of the protocols.

In accordance with prevailing data and concepts, the interpretation of the intranuclear inclusions as the result of an infectious process is tempting. This theory is favored by the fact that in general the deprivation of colostrum leads to a variety of infectious diseases, and that any evidence that this deprivation causes a recognizable deficiency disease is thus far lacking. If the process is infectious it could best be assumed as due to a filtrable virus occupying the nucleus and causing the changes leading to the concretions. Moreover, like the other infec-

<sup>12</sup> Van Leersum, E. C., *J. Biol. Chem.*, 1928, 79, 451.

tious diseases brought to the surface by the colostrum experiments, it would have to be regarded as of low virulence, appearing only sporadically under the usual environmental conditions and perhaps very rarely in older animals. Since the subject of spontaneous renal diseases of lower animals is still in the descriptive stage, we must await further developments and the increased prevalence of this type of disease which will permit a wider, more detailed study.

Another quite different theory to account for the renal lesions is the early absorption of *B. coli* toxins from the digestive tract. Most of the animals whose protocols are given went through an early attack of liquid stools. Those that die in this stage (scours) have intensely congested kidneys. The effect of the *B. coli* toxins eliminated through the kidneys may in certain surviving cases bring about the peculiar epithelial abnormalities described. To prove or disprove this hypothesis, any more definite experimentation than the one followed (withholding colostrum, etc.) cannot be carried out because the calf after the first week cannot be infected with *B. coli* by feeding. The intravenous injection of filtrates is not a complete substitute since the toxins formed in the ileum may be either more potent or different from these in the culture tube.

Intermingled with cases of the renal disease were those in which convulsive seizures coming on suddenly and accompanied by various forced movements indicating irritation of the central nervous system were the chief symptoms. These are twitching of one ear or tail, movements of the legs, staggering gait, difficult swallowing, disturbance of vision, grating of teeth, convulsion and death within one half to several hours after the sudden beginning of the attack. The postmortem changes are hemorrhages in the intrathoracic portion of thymus, petechiae on the epicardium and large vessels, and pulmonary congestion. In 6 animals thus affected, only one (No. 1416) did not die within a few hours of the beginning of the attack. This animal became blind and lingered along for 47 days after the attack when it was killed. Various causes suggested themselves, among them botulism, status lymphaticus, acute poisoning, and anaphylactic reactions. In two of these cases intranuclear bodies were present. The rest had not been examined as thoroughly for such bodies, since the earliest cases were regarded as due to poison. Tentatively the hypothesis is put forth

that this group of symptoms may be the earlier acute fatal stage of the renal disease and that the inclusion bodies develop in the less acute type. Or, a similar intranuclear affection of epithelial cells elsewhere in the body thus far overlooked, may lead to the convulsive type.

#### CONCLUSION

Renal lesions chiefly the result of obstructive processes were associated with intranuclear bodies in the epithelium of straight and convoluted tubules. In one animal these bodies were found in large numbers in otherwise still normal organs. The affected animals had been fed cow serum in place of colostrum.

#### EXPLANATION OF PLATES

##### PLATE 9

FIG. 1. Frozen section of fresh kidney of Calf 1532 in physiological salt solution, showing the almost universal presence of intranuclear bodies in the straight tubules.  $\times 205$ .

FIG. 2. Film prepared by scraping the cut surface of the fresh kidney, drying the film, passing it through absolute alcohol and water, staining in hematoxylin and eosin, clearing, and mounting in balsam.  $\times 560$ .

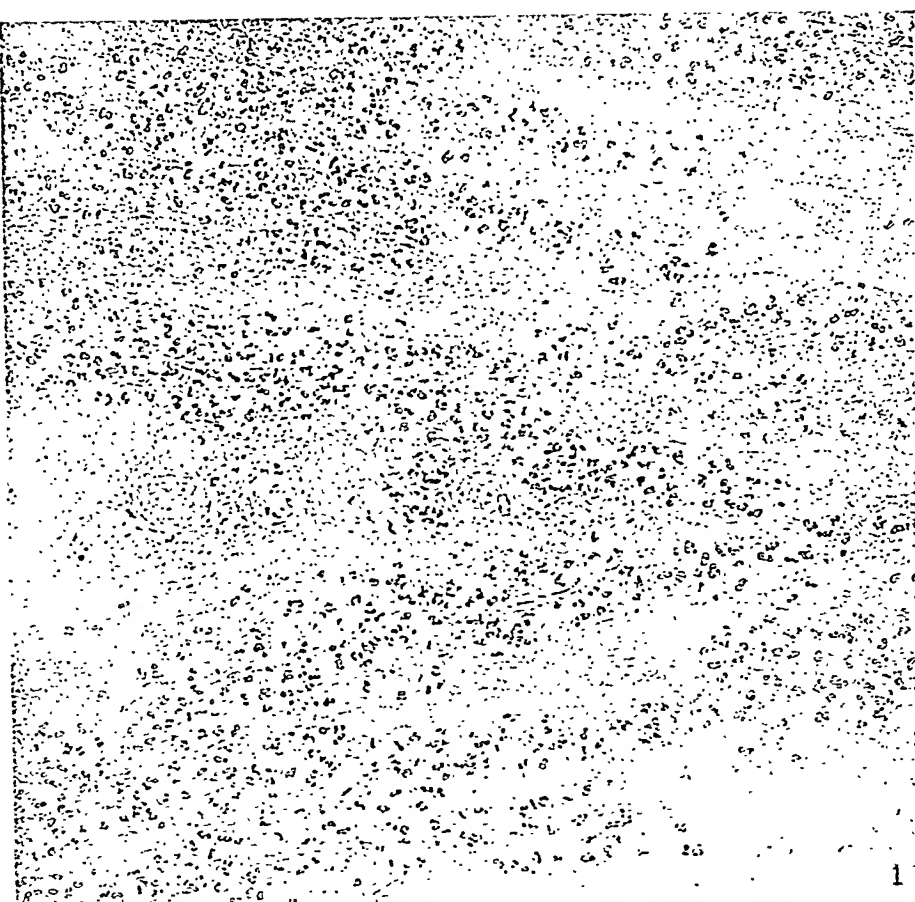
FIG. 3. The same preparation.  $\times 1920$ .

##### PLATE 10

FIG. 4. Frozen section of formalinized kidney of Calf 1511. Mounted in water.  $\times 200$ .

FIG. 5. Frozen section from the same kidney, showing the intranuclear site of the inclusion bodies.  $\times 350$ .





1

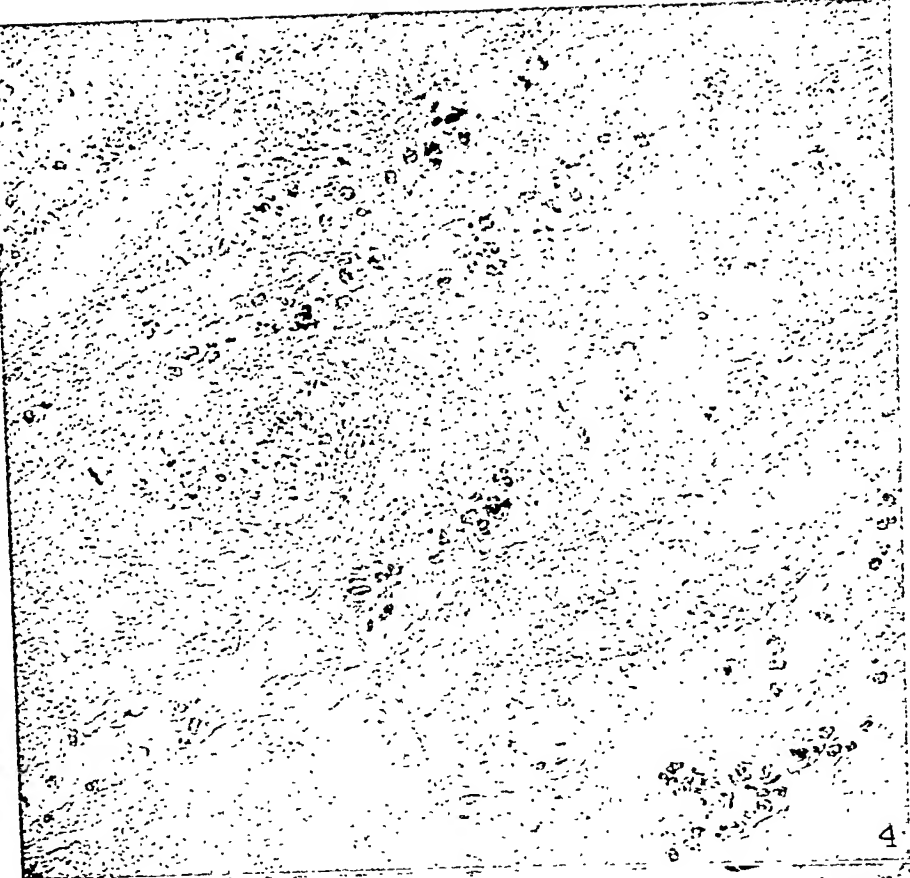


2



3









## STUDIES ON VITAL STAINING

### IV. INDIA INK AND BRILLIANT VITAL RED. IMPORTANCE OF CONSIDERING LIVER EXCRETION IN THE STUDY OF "BLOCKADE OF THE RETICULO-ENDOTHELIAL SYSTEM"

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#### INTRODUCTION

In previous papers of this series we have presented experiments dealing with the distribution in the body of certain dyestuffs when given intravenously (1). The two dyes, brilliant vital red and Niagara sky blue, like many other acid dyestuffs are gradually taken up by phagocytic cells in various parts of the body ("reticulo-endothelial system" of Aschoff). They differ from many dyes of this group in being taken up rather slowly by these cells, so that the concentration of dye in the plasma may be followed over a period of many hours before the concentration becomes too low to be determined by colorimetric methods. At first the tissues remove either dye quite rapidly from the blood stream, but later on as the tissues become more deeply stained they become less active, and at such concentrations of dye in plasma they take up dye less readily than they normally would. This sluggishness or "inhibition" of tissue activity represents in reality an equilibrium in dye concentration between tissues and body fluids, for if more of the dye be injected the tissues will take up much of it. The dye continues to pass into the tissues until a second equilibrium point is reached at a higher level.

These two dyes are rather similar in their general physico-chemical properties and we need not be surprised that they are deposited within the same identical phagocytes (2). Our evidence shows (2) that the presence of one dye within these cells does not prevent the cells from taking up the other at the normal rate. Furthermore, the phagocytes can take up both dyes simultaneously when they are injected together. The cells respond without hesitation to this double task, with the result that each dye is removed from the plasma with normal speed, not only at first when the cells are free of dye, but later on as well, when the cells contain considerable amounts of each dye. The two dyes within the cell do not appear to exert any mutual antagonism which would slow up the rate of storing dye or hasten the onset of the equilibrium for either dye.

It is common knowledge that enormous numbers of phagocytes exist within the connective tissues and within the lymph nodes. These cells do not lie in intimate contact with the blood stream as do the Kupffer cells of the liver or certain phagocytic cells of spleen or bone marrow. It has never been clear whether or not cells of this latter type are really more actively phagocytic than the cells more distantly removed from the circulating blood. The fact that they take up foreign materials so promptly may merely be a correlary of their accessibility to the blood stream and hence to the source of the foreign materials. At a later date we shall present data to show that after removal of liver or spleen, brilliant vital red is still quite rapidly removed from the circulating blood. The removal of the liver with all of its Kupffer cells or the spleen with its numerous phagocytes does not appear to cause great impairment of the phagocytic system as a whole. We may concede that as individuals the phagocytes of liver or spleen are perhaps more highly endowed types of phagocytes, still it is our belief that the phagocytes of the connective tissues generally are so extremely numerous that much and probably most of the total phagocytic power in the body resides in them.

When India ink is injected into the blood stream the particles of carbon tend to agglutinate, and very few of them succeed in passing through the capillary walls. They are promptly taken up and stored in those phagocytes which lie in intimate contact with the blood stream. Such injections offer us an opportunity to test our ability to "block" these particular cells without in any way involving the phagocytes which lie well outside the vessel walls. To test the phagocytic response of these ink-laden cells one should inject some second foreign substance which would be taken up by these cells, and by them alone. Unfortunately most if not all substances of this class are in such an unstable state of dispersion that they precipitate out in the blood stream. Many of these particles are caught and held mechanically in the capillaries, so that speed of removal cannot be used as a measure of phagocytic activity. On the other hand most dyestuffs, like brilliant vital red, reach vast numbers of phagocytes besides those which contain ink. Our experiments with liver removal and with splenectomy have lead us to believe that these extravascular cells possess a large total phagocytic power. We could hardly expect the presence of ink in the smaller group of cells to reduce the total bodily response very markedly, even though the ink did block the cells which take it up. For these reasons we were not prepared to find that India ink does actually delay the rate at which brilliant

vital red leaves the circulating blood. At first we seemed compelled to attribute this to defective phagocytosis, but later on we found that the ink injection inhibits the excretion of dye into the bile. The longer retention of dye in the plasma can be explained by defective elimination on the part of the liver.

From these observations it became evident that peculiarities of liver excretion demand careful attention. It is necessary to have accurate data on this subject if we are to interpret any data on the rate at which dye leaves the blood stream. Obviously, changes in this rate cannot be ascribed to changes in phagocytic response unless we can show that liver excretion is not responsible. Much of the literature of "blockade" does not take these considerations into account. It is not sufficient to assume that liver excretion is constant or that it is proportional to dosage of the dye. In fact this latter supposition is definitely contrary to fact (3). With small doses as much as 30 per cent may be eliminated into the bile during the first day, whereas with larger doses the percentage output may not be more than 15. After chronic vital staining or after single large injections the dye may be less efficiently removed from the blood stream, but as in the case of India ink injections, this must be attributed to the inefficient liver excretion and not to "blockade" of phagocytes.

### *Methods*

Details regarding care of the dogs and the collection and analysis of blood samples have been given elsewhere (1).

The India ink used was Higgins' American India ink. The exact composition of this ink has never been published. It contains about 10 per cent of solids. We have found that the carbon can be precipitated with dilute acids. If this black mass be resuspended in water and again precipitated with dilute acid and then washed with alcohol and dried it will be found to represent about 7 per cent of the original ink. This figure may be a trifle too high but we feel that most of the impurities have been separated from the carbon by this process of precipitation and washing. Kjeldahl determinations show that the precipitated mass contains almost no nitrogen.

The ink was injected either without dilution, or in some cases after dilution with an equal volume of normal saline. Since much of the ink is precipitated in the blood stream and is promptly filtered out by the capillaries we have not been interested in following the

rate at which the ink leaves the blood stream, for such studies give no information regarding the rate of true phagocytosis by the tissues. We may note in passing that in our experiments the blood stream was regularly free of carbon particles within an hour or less. We have taken advantage of this fact and have timed the administration of ink in such a way that ink would never be present in the plasma to interfere with the regular colorimetric determinations of dye.

Bile was collected for study in certain of the dogs.

A slight modification of the bile fistula operation of Rous and McMaster (4) has been made by Smith, Groth and Whipple (5) and their technic was used in the present experiments. By this method bile is collected by a cannula inserted into the common bile duct and is brought to the outside and collected in a rubber bag from which it can be removed daily, or oftener if desired. The rubber bag is protected from injury by suitable dressings which are placed about the dog. Bile can be collected whenever desired over a period of many weeks. The dog remains in good condition throughout unless pathogenic organisms gain entrance from the outside to the bile passages, but under favorable conditions, using aseptic technique in making the bile collections, the bile may be kept sterile for a number of weeks.

The quantitative analysis of brilliant vital red present in the bile collected offers certain difficulties. We had hoped that it would be possible to make estimations by means of the spectrophotometer, using a previously described method (6) for the quantitative analysis of colored mixtures. To investigate this possibility artificial mixtures of bile and brilliant vital red were made, but it was found that the absorption curves of the two substances were not strictly additive, though approximately so in certain portions of the spectrum. There was the further complication that the absorption bands of these two substances overlap to a very considerable extent. Both absorb light in the green and blue much more than in the red. Although the absorption curves for the two substances are by no means identical, yet the relations are such that no great precision can be expected by spectrophotometric methods. For these reasons we have adopted the well known and simple method of analysis based on the use of the "color comparator."

Two series of standard solutions are prepared, one containing bile in varying concentration from 1 to 10 to 1 to 60, the other containing brilliant vital red vary-

ing from 1 to 15 mg. per liter. The unknown is diluted to a varying degree but usually to about 1 to 30 and is poured into a test tube and placed in a rack behind another tube containing water. The light transmitted through these two tubes is then matched in quality and in intensity with that produced by placing in alignment two of the standard dye and bile tubes. By making successive trials a combination of a dye and a bile tube can be found which matches very closely the unknown, both as regards shade and intensity. Obviously, care must be taken that all of the tubes used are of the same diameter. The method is quick and simple and when dye is present in the bile in considerable quantities—200 mg. per liter or more—the error is somewhat less than 10 per cent, as can be shown by test analyses of known mixtures of dye and bile. The physiological changes which we will discuss are several times this experimental error, so that the method of analysis answers fully the present requirements.

#### EXPERIMENTAL

Brilliant vital red injected into a dog by the intravenous route disappears from the circulation at a rate which varies slightly with different individuals, but we have numerous experiments to show that for a given dog the experiment may be repeated at intervals of weeks or months with almost identical results. The elimination rate for the dog may be determined by a preliminary test dose of dye, and after several weeks the tissues will be almost entirely free of the latter and we are at liberty to repeat the experiment either with or without the introduction of procedures calculated to influence this established disappearance rate of dye.

*Dog 24-74. Shepherd. Weight 32 kg. (See Table 41.)*

*February 3, 1928. Control experiment to determine normal elimination from the blood stream of brilliant vital red.* Twenty cubic centimeters of an aqueous 2 per cent solution of brilliant vital red were injected into the jugular vein. At varying intervals thereafter samples of blood from the opposite jugular vein were taken into oxalate solution, and after centrifugalization the concentration of dye in the supernatant plasma was determined by means of the spectrophotometer. The results are given in the second column of Table 41.

*March 25, 1928. Experiment to determine the effect of ink injection on the disappearance rate of dye.* At 9:52 A.M. 20 cubic centimeters of 2 per cent brilliant vital red were injected. The 5 minute sample was taken at 9:57 A.M. and immediately following this, 10 cc. of Higgins' American India ink were injected into the jugular vein. The ink disappeared rather rapidly from circulation and the blood samples taken 1 hour later and subsequently contained no ink whatever. The amount of dye in the various samples of plasma taken during this

elimination period of 4 days is shown in the third column of Table 41. At no time during or following the injection of the dye or ink was there any evidence of sickness on the part of the dog.

May 20, 1928. Dye injection to test elimination rate 2 and 6½ months after injection of India ink. The experiment was carried out as on February 3. The results are shown in the fourth and fifth columns of Table 41.

TABLE 41

*Dog 24-74. Effect of Ink on Disappearance of Dye from Plasma*

Time after dye injection	Brilliant vital red in 1000 cc. plasma			
	Dye without ink	Dye with ink*	Dye 2 months after ink	Dye 6½ months after ink
	mg.	mg.	mg.	mg.
5 min.	260	260	240	255
1 hr.	225	225	225	215
6 hrs.	145	160	160	155
24 hrs.	37	114	72	43
48 hrs.	18	55	26	17
72 hrs.	8	32	16	13
96 hrs.	4	11	5	3

\* Ink 5 mins. after dye injection.

TABLE 42

*Dog 24-96. Effect of Ink on Disappearance of Dye from Plasma*

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 4½ months after ink
	mg.	mg.	mg.
5 mins.	480	460	460
1 hr.	335	460	390
6 hrs.	310	350	315
24 hrs.	160	240	180
48 hrs.	74	140	98
72 hrs.	57	91	54
96 hrs.	31	81	48

\* Ink 5 mins. after dye injection.

*Dog 24-96. Shepherd. Weight 15 kg. (See Table 42.)*

February 17, 1928. Control to determine rate at which brilliant vital red normally leaves the blood stream. Twenty cubic centimeters of 2 per cent dye injected into jugular vein. Samples of blood taken at intervals during the next 4 days

were received into isotonic sodium oxalate (1.6 per cent) solution. Each sample was promptly centrifuged. The amount of dye in the supernatant plasma in each case is shown in the second column of Table 42.

*May 27, 1928. The effect of an injection of India ink upon the disappearance rate of the dye.* As in the case of dog 24-74, 20 cc. of brilliant vital red were injected and 5 minutes were allowed for admixture with the circulating blood. Immediately after the 5 minute sample of blood had been taken for dye analysis, an injection of 10 cc. Higgins' American India ink was made. That the ink particles were rapidly swept out of the blood stream is shown by the fact that a blood sample taken an hour later contained no trace of carbon. The rate of dye elimination from the blood is indicated by the figures shown in column 3 of Table 42. There were no signs that either dye or ink were toxic to the animal.

TABLE 43

*Dog 25-29. Effect of Ink on Disappearance of Dye from Plasma*

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 4½ months after ink
	mg.	mg.	mg.
5 mins.	370	385	320
1 hr.	325	350	275
6 hrs.	205	320	215
24 hrs.	102	150	109
48 hrs.	50	94	61
72 hrs.	33	65	37
96 hrs.	17	48	20

\* Ink 6 hours before dye injection.

*October 7, 1928. Disappearance rate of dye 4½ months after injection of India ink.* The experiment is identical to that carried out on February 17. The concentration of dye in the plasma in each of the samples taken is shown in column 4 of Table 42.

*Dog 25-29. Collie. Weight 28 kg. (See Table 43.)*

*March 2, 1928. Control to determine the rate at which brilliant vital red normally leaves the blood stream.* Twenty-four cubic centimeters of 2 per cent brilliant vital red injected into the jugular vein. In the second column of Table 43 is shown the concentration of dye in each of a number of samples of plasma collected during the next 4 days.

*May 30, 1928. Effect of an injection of India ink upon the disappearance rate of the dye.* Unlike the experiments on dogs 24-96 and 24-74 the regular dose of 10 cc. Higgins' American India ink was given 6 hours before making the dye



injection. The latter consisted of 24 cc. of 2 per cent brilliant vital red, also given intravenously. No evidence of toxicity either on the part of ink or dye. Plasma collected at varying intervals following the injection of dye was analyzed for its dye content. The results are shown in the third column of Table 43.

*October 6, 1928. Disappearance rate of dye 4½ months after injection of India ink.* The experiment is similar to that performed on this dog on March 2. The concentration of dye in each plasma sample collected is shown in the fourth column of Table 43.

*Dog 27-243. Male collie. Weight 24 kg. (See Table 44.)*

*April 29, 1928. Control to determine the rate at which brilliant vital red normally leaves the blood stream.* Twenty cubic centimeters of 2 per cent aqueous brilliant

TABLE 44

*Dog 27-243. Effect of Ink on Disappearance of Dye from Plasma*

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink *	Dye with ink*	Dye 4 months after ink
	mg.	mg.	mg.
5 mins.	250	300	335
1 hr.	225	280	295
6 hrs.	155	205	185
24 hrs.	70	105	76
48 hrs.	30	74	26
72 hrs.	22	44	17
96 hrs.	11	37	6

\* Ink 24 hours before dye injection.

vital red injected intravenously. In column 2 of Table 44 are given data showing the concentration of the dye in the plasma in various samples subsequently taken.

*May 30, 1928. Effect of an injection of India ink upon the disappearance rate of the dye.* Unlike the other cases the 10 cc. dose of American India ink was given intravenously 24 hours before the dye was given. As in the control experiment one month previously, 20 cc. of the 2 per cent aqueous brilliant vital red were given. The plasma collected at varying intervals following the injection of dye was analyzed for its dye content. The results are shown in column 3 of Table 44. There was a short period of deep breathing and some nystagmus following the injection of ink. Prompt and complete recovery.

*October 7, 1928. Disappearance rate of dye 4 months after injection of India ink.* The experiment is identical to the control carried out on April 29. The results are shown in the fourth column of Table 44.

We have a number of experiments, all of which go to show that the intravenous injection of India ink slows up very markedly the rate at which brilliant vital red leaves the blood stream. We submit four such experiments (Tables 41-44 inclusive). The results of these experiments are in agreement and the outstanding features are averaged and shown graphically in Chart 4A. It will be observed that the initial blood sample taken 5 minutes after the injection of dye shows the plasma to contain nearly 350 mg. dye per liter plasma. In all cases the greater part of the dye leaves the blood stream during

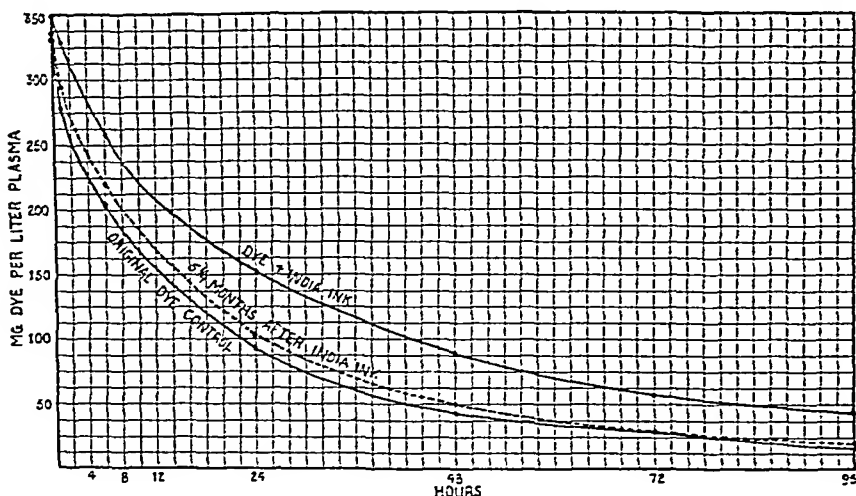


CHART 4A. Elimination of brilliant vital red from plasma. Effect of India ink. (Curves represent the averages of four dogs—Tables 41-44 included.)

the course of the next four days, but there is a most striking retardation in the elimination rate when a small dose of ink is injected at the beginning of the experiment. This retardation begins almost at once and at the end of 48 hours there is twice as much dye in circulation as in the normal control determinations, and later on the discrepancy is even somewhat greater. The change is several times what we know to be the maximum experimental error.

Large doses of India ink are somewhat toxic but the small doses used by us did not disturb the animal. In all cases they ran about in a normal manner and ate the regular ration of hospital scraps.

We have noted occasionally that there is some retardation in coagulation of blood following injection of ink, but hemorrhages into skin, mucous membranes or intestinal tract were never noted. We have abundant proof that the elevation of the dye elimination curve following ink is not due to a decrease in the blood volume, for the centrifuged samples of blood showed a constant ratio of cells to plasma throughout the experiment. Furthermore in two of the experiments, (Tables 43 and 44), the ink was given a number of hours before the dye was injected, and in neither case was the initial dye reading greatly elevated as one would expect if the ink had caused a reduction

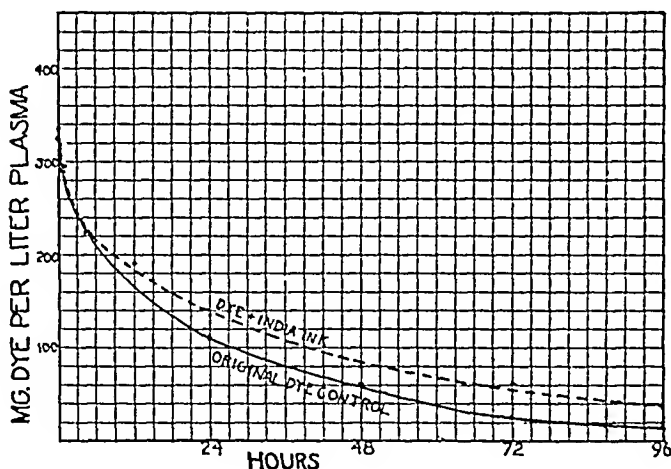


CHART 4B. Dog 27-229. Elimination of brilliant vital red from plasma. Normal control contrasted to curve showing ink given six hours after dye.

in blood volume. There can be no doubt whatever that the small dose of ink has brought about a true retention of dye in the blood stream. Another experiment showing this phenomenon is shown in Chart 4B. Here the administration of ink was delayed until 6 hours after the dye had been introduced into the blood stream. We note that during these first 6 hours the dye curve falls off at a rate which is almost identical to the control done 5 weeks previously, but from the moment the ink was injected we note that the two curves separate, indicating that the ink has acted very promptly in slowing up the disappearance rate of the dye.

*Dog 27-229. Weight 22.5 kg. (See Chart 4B.)*

*April 29, 1928. Control to determine the rate at which brilliant vital red normally leaves the blood stream.* Twenty cubic centimeters of 2 per cent dye were injected intravenously. There were no untoward symptoms following this procedure. The concentration of the dye in the plasma at different intervals is recorded in Chart 4B.

*June 2, 1928. Effect of an injection of India ink upon the disappearance rate of the dye.* Twenty cubic centimeters of 2 per cent dye were injected intravenously, but 6 hours after the dye, 10 cc. of American India ink were given. During the ten minutes following the ink the animal was dyspneic, his muscle tone diminished and for the rest of the day he seemed somewhat depressed. However, the next day he appeared quite well.

The concentration of the dye in the circulation at different intervals is shown by the dotted line in Chart 4B.

We call especial attention to the results shown in Table 44. In this case the ink was injected 24 hours before the dye was given. The ink caused only slight and very transient clinical disturbance. Of particular significance is the fact that we note well marked delay in the rate at which the dye leaves the blood stream. In fact the delay seems to be as great as when ink and dye are given on the same day. It is clear that the action of ink in retarding dye elimination persists for several days at least. When a test dose of dye was injected 5 months after ink injection (Chart 4A) the rate of dye elimination appears to be almost if not entirely normal. In fact we believe that recovery is practically complete in 3 weeks (Chart 4C) although the departure from normal at that time seems to be slightly more than we can charge to experimental error. We have other experiments which show retardation 3 weeks after ink even more distinctly than this; still we feel that the effect of the ink largely wears off after the first week.

*Dog 28-165. Mongrel. Weight 15.5 kg. (See Table 45.)*

*May 11, 1929. Control to determine rate at which brilliant vital red normally leaves the blood stream.* Fifteen cubic centimeters of 2 per cent dye injected into jugular vein. At intervals during the next 4 days samples of blood were drawn into isotonic sodium oxalate and after centrifugalization the amount of dye in the plasma was determined. The results are shown in the second column of Table 45.

*June 1, 1929. The effect of an injection of India ink upon the disappearance rate of the dye.* As on May 11, 15 cc. of 2 per cent brilliant vital red were injected

into the jugular vein. Five minutes were allowed for complete admixture of the dye with the circulating blood and at the end of this time a sample was taken for analysis. Immediately thereafter 8 cc. of Higgins' American India ink were injected. Dog was very slightly depressed for about an hour only. No vomiting.

TABLE 45

*Dog 28-165. Effect of Ink on Elimination of Dye from Plasma*

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 3 weeks after ink
	mg.	mg.	mg.
5 mins.	520	455	495
1 hr.	430	390	395
6 hrs.	245	300	305
24 hrs.	160	165	160
48 hrs.	69	110	68
72 hrs.	43	69	43
96 hrs.	28	43	30

\* Ink 5 mins. after dye injection.

TABLE 46

*Dog 28-184. Effect of Ink on Elimination of Dye from Plasma*

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 3 weeks after ink
	mg.	mg.	mg.
5 mins.	410	380	375
1 hr.	340	370	325
6 hrs.	210†	275	230
24 hrs.	96	165	109
48 hrs.	45	86	49
72 hrs.	39	45	24
96 hrs.	39	28	16

\* Ink 5 mins. after dye injection.

† By interpolation.

Samples of blood taken an hour after these injections as well as those taken later contained no dye in the plasma though smears showed some carbon in the white cells. The dye concentration was determined at intervals during the following 4-day interval. The results are shown in the third column of Table 45.

*June 22, 1929. Disappearance rate of dye 3 weeks after injection of India ink.*

The experiment is identical in technical details to that carried out on May 11. The concentration of dye in the samples taken for analysis is shown in the fourth column of Table 45.

*Dog 28-184.* Mongrel, male. Weight 16.5 kg. (See Table 46.)

As regards dates, dosage and the taking of samples this experiment is in every way identical to that on dog 28-165. The details are given in the protocol of that experiment. As in that experiment there was slight transitory clinical disturbance following the injection, but later on in the day the dog ate well and was normally active. The dye readings in the plasma are given in Table 46.

The fact that an injection of India ink will retard the rate at which brilliant vital red leaves the blood stream might be thought to prove

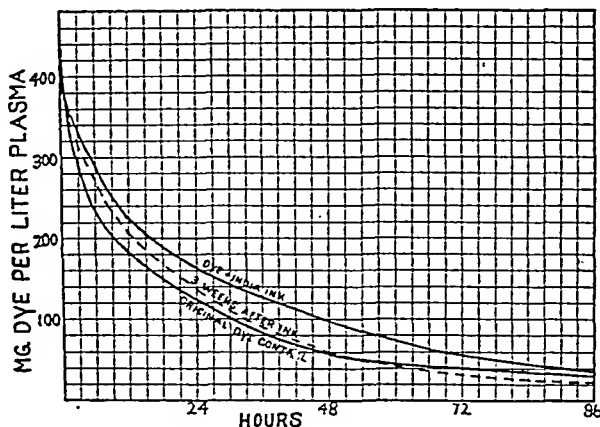


CHART 4C. Elimination of brilliant vital red from plasma. Effect of India ink. Almost complete recovery shown within three weeks. (Curves represent the averages of two dogs—Tables 45 and 46.)

that the ink-laden phagocytes were partially blocked against the entrance of the dyestuff. Indeed, this was our original view. The evidence seems more convincing than much of that commonly offered to support the blockade theory. Our doubts were aroused in the course of studies which we were carrying out on bile fistula dogs. In the course of these experiments it was found that India ink possesses a remarkable ability to slow up or prevent the passage of the dye into the bile. In some cases this effect was associated with considerable diminution in the total volume of bile output per day,

but in many cases the normal amount of bile was excreted. Ordinarily 5 or 10 cc. of ink were injected, and as a rule about twice this amount of two per cent brilliant vital red. In all cases it was clearly seen that for 24 or 48 hours the bile recovered from the animal was of the normal greenish yellow color, quite in contrast to the orange or cherry red colored bile obtained when dye alone was injected. Obviously, such dye retention within the body may well account for the longer retention of dye in the plasma.

In Tables 47 and 48 we present the results of two experiments of the sort just described. In the first, only 100 mg. of dye were injected but we noted that in the normal control period the bile was brightly colored for some days. More dye was eliminated in the first 24 hours than later on, but considerable amounts are present for some time. In all, about half of the dye was recovered in the course of the first 6 days. In the second half of the experiment ink was given along with the dye, and here we noted almost complete failure of dye excretion during the first 24 hours; nor is the inhibition entirely transitory, for the total excreted during the first 4 days is less than half the amount normally excreted in the first 24 hours. Despite this remarkable inhibition in dye excretion we observed no evidence of toxicity on the part of the ink or dye; in fact the dog ate normally throughout the experimental period and the normal amount of bile was excreted. We have analyses to show that in such cases the total output of bile pigments and bile salts also approximates the normal, though the results are somewhat variable. These facts suggest that there is no overwhelming injury to the liver. It would appear that there is a dissociation of functions, for ink seems to inhibit dye excretion without necessarily affecting certain other well established functions. Experiment 32 (Table 48) differs from this experiment in minor respects only. The amount of dye given was three times as great, and as a result we note rather large amounts of dye excreted in the control period. Again, the effect of ink is to suppress the elimination of dye into the bile. The suppression is practically complete for 48 hours, and along with this we note that the daily volume of bile is markedly reduced. Later on the volume of bile returns to normal or slightly above normal, and we observe that some dye is being eliminated, though this activity is rather transitory. In this

case nearly 85 per cent was eliminated during the 9 day control period, but influenced by ink, the liver secreted only one-fourth as much in a similar period.

The dye thus retained within the body becomes distributed between plasma and tissues, coloring each more intensely than normal. We see in these observations a ready explanation for the fact that following an ink injection the dye leaves the blood stream much more slowly than normal, and we feel it unnecessary to suppose that ink has impaired the capacity of the phagocytic system for storing dye.

TABLE 47

*Experiment 31. Effect of 5 Cc. India Ink on Excretion of Brilliant Vital Red*

	Volume of bile eliminated	Dye per 1000 cc. bile	Total 24 hour dye output
	cc.	mg.	mg.
Dye (100 mg.) injected with- out ink			
1st day	85	240	20
2nd day	75	140	11
3rd day	80	120	10
4th day	75	40	3
5th day	60	30	2
6th day	80	15	1
Dye (100 mg.) injected along with ink (5 cc.)			
1st day	85	0	0
2nd day	85	40	3
3rd day	85	40	3
4th day	75	40	3

Dog is a 13 kg. female terrier.

We have been concerned with the question of how it is that ink so completely prevents the excretion of a dye by the liver. There is little to indicate that ink is a liver poison, for we often see apparently normal elimination of bile pigments and bile salts despite the inability of the liver to excrete dye for several days, and with these small doses of ink the dog gives little or no external evidence that the ink is toxic. Work still in progress seems to negate our earlier supposition that the particles of carbon are responsible. We hope to report our



findings more fully in the future, but the evidence seems to indicate that other substances within the ink may be to blame. Finely ground graphite suspended in acacia may be injected without impairing the ability of the liver to excrete dye. Unless such mixtures are made rather strongly alkaline they are quite unstable and the particles are

TABLE 48

*Experiment 32. Effect of 7.5 Cc. India Ink on Excretion of Brilliant Vital Red*

	Volume of bile eliminated	Dye per 1000 cc. bile	Total 24 hour dye output
	cc.	mg.	mg.
Dye (300 mg.) injected with- out ink			
1st day	100	400	40
2nd day	120	400	48
3rd day	110	360	40
4th day	125	240	30
5th day	110	240	26
6th day	130	200	26
7th day	115	160	18
8th day	130	120	16
9th day	120	80	10
Dye (300 mg.) injected along with ink (7.5 cc.)			
1st day	31	0	0
2nd day	23	0	0
3rd day	56	20	1
4th day	140	40	6
5th day	140	120	17
6th day	140	80	11
7th day	140	80	11
8th day	140	80	11
9th day	130	40	6

Dog is a 13 kg. female terrier.

rather large, but we have observed that the particles are distributed about the body in the same manner observed after India ink injections and in each case the Kupffer cells of the liver are heavily laden with carbon particles. These experiments suggest strongly that the inhibitory effects of India ink must be sought in elements other than in the carbon.

This conclusion is reinforced by several experiments in which we have precipitated the carbon from India ink and have observed that the brownish colored fluid so obtained was effective in inhibiting dye excretion by the liver. The carbon of the ink was precipitated by the addition of a known amount of dilute hydrochloric acid and after centrifugalization the supernatant fluid was kept and later combined with certain brownish soluble ingredients which can be extracted from the mass of carbon with the aid of alcohol and of dilute acid. The alcohol was evaporated off and the various carbon-free fractions combined, and enough standard sodium hydroxide added to restore the original alkalinity. As in the case of India ink this solution may be given intravenously without producing any external manifestations of toxicity, but dye elimination by the liver is almost as completely inhibited as in the case of the original untreated ink. It is well known that India ink is rather strongly alkaline. Titration shows that it is neutralized by an approximately equal volume of  $N/14$  hydrochloric acid. We have injected 10 cc. of  $N/14$  sodium hydroxide into the blood stream without observing any decrease in the rate at which the liver will excrete brilliant vital red, simultaneously injected. We do not believe that the alkalinity of the ink is responsible for the inhibition observed with ink injections.

#### SUMMARY

When brilliant vital red is injected into the blood stream of dogs much of it is slowly taken up into numerous phagocytes scattered throughout the tissues ("reticulo-endothelial system" of Aschoff).

The rate at which the dye leaves the blood stream is determined in large part by the action of these phagocytic cells, but the excretion of dye into the bile is also in part responsible for the loss of dye from the plasma.

The injection of a small amount of India ink into the blood stream results in lowering the rate at which the dye disappears from circulation. The fact that much of the carbon of the ink is promptly taken up by the phagocytes would lead one to suspect that they were saturated with foreign materials, or "blocked" against the entrance of dye, but it is shown that the ink causes a remarkable inhibition of the excretion of dye into the bile, and this alone seems to account for

the longer retention of dye in the blood stream. There is no evidence that any of the retention is due to defective activity on the part of the phagocytes.

Thus, prolonged retention of foreign materials in the blood stream cannot be cited to prove "blockade of the reticulo-endothelial system" unless one can rule out such peculiar reactions on the part of excretory organs. It is felt that the literature of "blockade" should be studied with such sources of error in mind.

Preliminary studies indicate that the suppression of dye excretion by the liver is not due to the carbon content of the ink. Studies of other components of the ink are now in progress.

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## STUDIES ON VITAL STAINING

### V. DOUBLE STAINING WITH BRILLIANT VITAL RED AND NIAGARA SKY BLUE. CORRELATION OF HISTOLOGICAL WITH PHYSIOLOGICAL DATA

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#### INTRODUCTION

After repeated daily intravenous injections of brilliant vital red, the skin, the connective tissues and various internal organs of dogs take on a deep red coloration. This color is due in small measure to the diffuse staining of certain non-cellular elements, particularly of the elastic fibers, but in greater part to the deposition within certain cells of great numbers of fine granules of the dye. The staining of elastic fibers, though slight, is well seen by gross inspection of the walls of the larger arteries. Microscopic examination shows quite clearly that the elastic fibers so abundantly present are diffusely and uniformly tinged with dye, there being no evidence that the dye is deposited in granular form within these fibers. Bouffard (1), Goldmann (2), Schulemann (3), Pappenheim and Nakano (4) and many others have observed the diffuse staining of elastic fibers with trypan blue, though pyrrol blue gives no such staining (4), nor does lithium carmine (5) though both of these dyes are excellent "vital stains." Occasionally one can see that brilliant vital red has tinged the delicate ramifying elastic fibers present in the areolar connective tissues but a glance through the microscope shows that the dye, like the other dyes just mentioned, is stored largely in granular form in the phagocytic cells to which we have referred. This segregation of dye within living cells has been the subject of innumerable investigations. For many of the details we refer to the reviews by Möllendorff (6), Aschoff (7), and Börner-Patzelt, Gödel, and Standenath (8).

Amongst the most active of these phagocytic cells are the Kupffer cells of the liver. Unlike many of the cells of connective tissues they lie in intimate contact with the blood stream and are directly exposed to foreign substances introduced into the circulating blood. Thus it is that these cells can take up India ink introduced into the blood whereas the phagocytic cells of the connective tissues generally have little opportunity to take up carbon particles which escape with such difficulty

through the capillary walls. Other cells like those which line the sinusoids of the spleen also have direct access to ink or dyes which may be present in the circulating blood. The endothelial cells of certain capillaries in the cortex of the adrenal and in the bone marrow also have phagocytic properties and may take up vital dyes, but the endothelial lining of blood vessels elsewhere seems to be almost entirely devoid of ability to retain these foreign substances.

Aside from these specific and well localized phagocytic types of endothelium, one finds in the connective tissues themselves enormous numbers of cells which are capable of taking up and storing in granular form a great variety of foreign substances which are sufficiently diffusible to pass through the capillary walls. A vast group of workers have shown that these cells of the connective tissues vary considerably in their morphology and there is still much uncertainty regarding the derivation of many of them.

Although many of the cells of connective tissues show little avidity toward acid colloidal dyes, still the majority can be stained under proper conditions of dosage and length of staining. Mast cells, lymphocytes and ordinary leukocytes show almost no tendency to take up these "acid dyes," but in the connective tissues are a vast number of "macrophages," "tissue phagocytes," "histiocytes," "clasmatocytes," or "resting wandering cells" as they are variously called, and these cells are highly phagocytic. They will take up particles of carbon or other finely granular material if this is injected directly into the tissues, but even more interesting is their capacity to take up various dyestuffs which are injected locally or which diffuse from the blood stream into the tissue fluids. In building up these intracellular granules, the dye is taken from solution and is greatly concentrated within the cell. It is obvious that the dye is not held there in simple solution, for the concentration is clearly many times that of a similar globule of a saturated solution of the dye. In the case of a few of the acid dyes, needle-like crystals may be formed but in no case is the exact nature of this concentrating mechanism understood. The literature of this question has been reviewed by Möllendorff (6). It remains an open question whether this process of dye storage differs fundamentally from the phagocytosis of finely particulate matter by these same cells. The former has been referred to as "dypsocytosis" (9) but this term has not gained wide acceptance. In the present experiments we have used the term "phagocytosis" to cover both. We have also used the term "vital staining" to refer to the dye storage, despite the fact that the word "staining" would seem to infer a reaction between dye and protoplasmic granules or other cytoplasmic constituents—a point still very much in dispute in the case of the "acid" dyestuffs.

Aside from these macrophages which are capable of ameboid motion, the fibroblasts of the connective tissues show an interesting behavior toward these acid vital dyes. Concerned as they are with the formation and maintenance of the fibers which bind the parts together, these cells have a much underdeveloped ability to take up foreign substances from the surrounding fluids. The faint

granular storage of lithium carmine by these cells is well shown in the plates of Ribbert (10) and Kiyono (11). Goldmann (2) showed that they will take up pyrrol blue. Evans and Scott (9) have placed particular emphasis upon the fact that they take up the dye much later than do the macrophages, and they have shown that granules do not appear in the fibroblasts until the animal has received large quantities of the dye. This sharp distinction in reaction has led Evans and Scott to set them aside as a distinct physiological class of cells.

These morphological details give us much valuable information concerning important physiological reactions of the tissues—reactions which would pass unobserved were it not that the tintorial properties of these dyestuffs make their recognition easy. There is every reason to suspect that many products of normal metabolism in the body may be distributed in analogous ways, though they lack the color properties necessary for their demonstration.

It is to be regretted that the morphological studies with dyestuffs have always been so purely qualitative. It is most desirable that we know more about their distribution in the tissues from a quantitative point of view. Unfortunately histological methods are almost purely qualitative and chemical extraction of ingested dyestuffs is beset with serious difficulties.

At the present state of our knowledge it is perhaps as well that we restrict our quantitative studies to the phagocytic response of the body tissues as a whole. Adequate means for such studies exist, for, with certain restrictions (12), the rate at which a dye leaves the circulating blood stream is a measure of this tissue response, representing as it does the sum total of the absorptive power of the elastic tissue and of all of the "macrophages" and other phagocytic cells previously mentioned. The reason that this quantitative mode of attack has been neither popular nor successful in the past must be that many dyestuffs are not well suited for such studies. Many of them leave the blood stream in large part before there has been time for them to be thoroughly mixed with the circulating blood. Under these circumstances efforts to follow the rate of elimination from the blood stream must necessarily fail. Other substances, like India ink and many dyestuffs are precipitated by the body fluids, and the particles are mechanically caught and held for a time in certain of the capillaries. Obviously, here too, the rate of disappearance from the blood is no criterion of the phagocytic response of the tissue. With other dyes the elimination through the urine or the bile is so rapid that the tissues are afforded little opportunity to display their phagocytic properties, and here again the elimination rate gives none of the desired information concerning tissue absorption. Fortunately, certain dyestuffs are relatively free from these defects, and from this group we have selected two, brilliant vital red and Niagara sky blue, as being excellent representatives. A number of the physiological studies have been reported in the previous papers of this series. The results of those experiments will be reviewed in the light of the morphological studies about to be reported.

### *Methods*

Many of the preliminary studies were made on rabbits, but typical experiments of all classes were made on dogs also, and these latter experiments are of particular interest because of the close relationship existing between them and the physiological experiments on distribution and elimination of dye and ink reported in previous papers of this series. In the case of both dogs and rabbits the ink was always given intravenously, but the rabbits in most cases received the dye into the peritoneal cavity, from which it is rapidly absorbed and carried with the circulating fluids to all parts of the body. The dyes and ink were given to the dogs by the intravenous method. The dogs received the regular mixed diet of hospital table scraps; the rabbits received hay, oats and cabbage.

The distribution of India ink can be studied to advantage in fixed tissues imbedded in paraffin and cut and stained by any of the ordinary methods. The two dyes, brilliant vital red and Niagara sky blue, are stored in granular form in many phagocytic cells scattered throughout the body, but the dye appears to be rather loosely fixed within the cell and after death it tends to diffuse, especially if the tissues are placed in any of the solutions ordinarily used in fixing and imbedding. Niagara sky blue suffers from this defect somewhat less than does brilliant vital red but in both cases the results in fixed tissues are quite unsatisfactory, and clear-cut pictures are to be obtained only by the study of freshly drawn films of tissues, teased preparations and fresh frozen sections of unfixed tissue. Even in these cases it is best to avoid adding water or saline to the preparation, and it is well to study the section immediately, without the addition of fluid or cover glass, though addition of glycerine and a cover-glass sometimes gives very satisfactory results. A drop of immersion oil may be placed directly upon preparations of omentum or mesentery and the tissue thus made available for study with the oil immersion lens.

It is desirable that several workers be concerned in making the fresh tissue preparations, for much valuable time may be saved and a maximum number of preparations may be prepared and examined before the cells die and the dye begins to diffuse away. Even so, it is rarely possible to make a detailed study of every organ while the tissues are fresh and much of the information is necessarily gained by piecing together the results of several similar experiments.

### EXPERIMENTAL

The dye granules formed within the cytoplasm of living cells present great variations in size, shape and arrangement depending upon the dye employed and upon the amount of dye administered and upon the rapidity with which the staining is brought about. Much of the earlier work has been reviewed by Möllendorff (6). The magnitude and complexity of this subject is well brought out in an excellent monograph by Evans and Scott (9). Their experiments cover a wide territory

and they have formed certain general conclusions regarding the effect of dosage and the duration of the treatment with dye. It is our hope that much detail still lacking may be filled in and that it may be possible to bring such morphological studies more closely into relation with the physiological aspects of dye storage. We feel that the morphological studies gain greatly in significance if carried out in conjunction with quantitative measurements of the rate at which the cells remove the dye from lymph or plasma. Our previous studies (12, 13) on the rate at which brilliant vital red and Niagara sky blue pass out of the blood stream into the tissues have not been extended in detail to other dyes, and for this reason it seemed desirable that we confine our microscopic studies to these two dyes.

We have found interesting cytological details which vary with the amount of dye and with the way in which it is given. Much of this work must be presented at a later time. At present we can consider only those findings most directly related to our previous studies on the rate at which these dyes leave the circulating blood.

*Experiment 22. Moderate Staining of Dog with Brilliant Vital Red.*

Dog 28-266. Brown terrier, 8.2 kg.

May 20-23 inclusive, 6 cc. 2 per cent brilliant vital red daily. Eight more on 24th and 6 cc. on morning of May 25. Animal killed with ether several hours later. Tissues moderately stained. Skin and connective tissues delicate pink. Lungs almost free of dye. Aorta moderately stained. Liver dark red. Bile thick and deep cherry red. Lymphatics at hilus of liver distended with clear reddish lymph. Spleen not enlarged but rather red and firm. Cortex of kidney moderately red. Medulla pale pink. Urine from bladder clear and yellow. Moderate staining of ileo-cecal lymph glands. Central two-thirds of each is bright red. Choroid plexus dark red, but meninges and brain unstained.

Microscopically, a bit of omentum spread out on a slide shows enormous numbers of macrophages. Careful inspection shows that they contain very large numbers of extremely fine pink granules. No conspicuous large clumps of dye in any of these cells. All these cells are stained very much alike and all have round or slightly oval nuclei. The cell bodies are irregularly globular. No dye in fibroblasts. No dye in endothelium of vessels, though elastic tissue of walls of certain vessels is pale pink. Many Kupffer cells contain moderate numbers of dye granules. No large dye clumps. Many convoluted tubules of kidney contain numerous fine dye granules in epithelial cells but no granules in part of cell next to lumen. Many granules about the nuclei or at the basal side of the cells. Hypophysis faintly pink, and frozen sections show numerous irregular triangular cells containing fine dye granules. They appear to be between the epithelial cells. No dye seen in the latter. Epithelial cells of choroid plexus contain numerous dye granules which are extremely fine and uniform in size. A few cells, apparently macrophages, between these cells contain more abundant dye.



*Experiment 23. Heavy Staining of Dog with Brilliant Vital Red.*

Dog 28-93. Fat female poodle, 8.0 kg.

April 24-May 1, 24 cc. 2 per cent brilliant vital red daily. Dog somewhat weak on last day. Centrifuged plasma obtained five minutes after last dye injection contained 1030 mg. dye per liter; six hours later 830 mg. Dog then killed with ether. Connective tissues deep red, especially the fascia of the muscles. Liver, spleen and kidneys deep red. Aorta and other large vessels deeply stained. Microscopically, elastic tissue of aorta is pink. Bits of mesentery and omentum spread out on slides show many macrophages varying somewhat in size, the largest ones being about 30-40 micra in diameter. The granules of dye which they contain are decidedly irregular and angular and variable in size. No crystal formation in any of the cells. Apparently the central portions of certain of the larger granules are a trifle paler than the peripheral portions. Epithelial cells of liver contain no dye but the Kupffer cells are much more numerous than normal and contain many large and small dye granules. Many small angular cells, apparently endothelial cells, in adrenals contain dye granules but no dye seen in the epithelial cells. Fibroblasts in connective tissue films and in mesentery and omentum have distinct oval nuclei and there are many small spherical uniform dye granules in the cytoplasm. Such granules are present also in the ramifying processes of these cells. This staining of fibroblasts is very faint.

*Experiment 24. Staining of Dog with Brilliant Vital Red. Examination of Connective Tissue Films from Thigh.*

Dog 28-281. Short-haired brown adult mongrel, 12.6 kg.

July 1-4 inclusive, 15 cc. 2 per cent brilliant vital red injected daily. Preceding the injection on July 4 an area on inner side of hind leg was shaved and with aid of novocain a small incision in skin was made and bits of connective tissue removed from between muscles. Film preparations covered with glycerine and cover-glass were examined with oil immersion lens. There are moderate numbers of dye-laden cells, most being rather thin and somewhat elongated, but not so much so as fibroblasts. Some cells contain more dye than others. All granules are rather uniform in size and none are extremely large. Most cells contain between 30 and 50 granules each.

July 6-9 inclusive, 15 cc. 2 per cent brilliant vital red injected daily. Films again examined on July 10. Preparations rather variable but rather clearly there are more dye-laden cells than on July 4. The enlargement of individual cells is very striking, most of them being several times as large as on July 4, and they are more rounded and many of them are much more than 30 micra in diameter. The dye granules are still uniform in size, though possibly somewhat larger. The increase in number of granules per cell is very striking. Many contain well over 100, and some more than 150. The intensity of dye in individual granules appears unaltered. No change in shape of dye granules.

No more dye injections made, but during following days skin and mucous membranes became more pale. Films again made on July 17. There appear to be

as many dye-laden macrophages as before, and dye-containing fibroblasts are possibly more numerous. They contain many fine granules of dye but these granules are quite small. The macrophages appear unaltered in size and the granules are certainly not less numerous but perhaps slightly smaller. Very clearly the granules vary more in size and many macrophages contain 1-3 large irregular granules or clumps of dye. No evidence of change in shade or quality of color of dye granules, though a few appear a little paler than before.

The general distribution of brilliant vital red is rather similar to that of dyes studied by Ribbert (10), Goldmann (2), Schulemann (3), Kiyono (5) and others. Experiment 22 will serve to illustrate the findings where the tissues are but moderately stained with this dye. In contrast to this experiment the tissues in experiment 23 were heavily stained. This was quite evident on gross inspection of various organs and tissues.

Histological study shows in each case the formation of dye granules in widely scattered cells. Most brilliant deposits are seen in the Kupffer cells in the liver, in the sinus endothelium of the spleen and in the phagocytes of the splenic pulp. We recognize also the well-known deposits in the sinus endothelium of lymph nodes, in the endothelium of the capillaries in the adrenal cortex, and above all the deposits of dye in the macrophages scattered throughout the connective tissues generally. As Evans and Scott have shown, the fibroblasts take up dye much more feebly than do the macrophages, but in experiment 23 so much dye had been injected that the fibroblasts show slight though definite granules of dye. This participation on the part of new cell types helps explain the ability of the body to handle ever increasing amounts of dye. There is also reason to believe that large or often repeated smaller doses of dye stimulate proliferation on the part of the more active types of phagocytes.

Investigators have repeatedly stressed the fact that with chronic vital staining one sees increased numbers of Kupffer cells in the liver sinusoids. This is entirely in accord with our own observations. It is difficult to estimate an increase in the number of macrophages in the connective tissues, for they are always irregularly scattered and it is difficult to get representative preparations. Nevertheless it does seem that some such increase does occur. Cell proliferation on the part of phagocytes represents an adaptation in time of

stress, and the dye storage by fibroblasts must also help to rid the circulation of these foreign materials.

We must recognize that the cell itself has remarkable powers of adaptation. Thus, the heavily stained animal (experiment 23) has much larger dye granules in the cells than does one which is feebly stained (experiment 22). Along with this increase in size of granules it appears that the individual cells increase somewhat in size.

We must confess that frozen sections and teased preparations of the internal organs are not entirely satisfactory for the study of fine cytological details, especially in the case of dyes which so readily diffuse out of the cells after death. It is much easier to get satisfactory preparations from the subcutaneous tissues of the thigh. Evans and Scott (9) have made beautiful films of such tissue and we can agree with them that with proper precautions one can make out the finest details regarding the shape, size and even the number of granules within the cells. Our observations on these tissues show that cell granules may increase markedly in number during the earlier stages of staining and as representative of these observations we may cite experiment 24. We note that in this experiment the increase in size of the macrophages is quite evident as soon as they begin to show considerable deposits of dye granules. In fact it would seem that the increase in size is almost proportional to the increase in the amount of dye within the cell. The result is that the dye granules seem to be no more closely packed than they were during the earlier stages of staining. It is noteworthy that in this experiment there is very clear evidence that the dye granules have increased greatly in number. It would seem that after certain granules had reached a certain size, new foci are formed, but our inability to find numbers of these newer and smaller foci would suggest that relatively few active centers of growth occur at any one time but that these centers grow rapidly and newer ones are then formed.

This experiment appears to be out of agreement with experiments 22 and 23 just cited. These pointed to the conclusion that many small granules are present at first and that they all enlarge to reach the size seen in the more heavily stained animal. We are unable to state the factors which determine the growth by accretion on the one hand or the growth by new granule formation on the other. We believe that both processes occur. Perhaps each mode is character-

istic of certain cell types, but slight differences in mode of administering the dye must also be considered. The importance of the time factor is evident from experiment 24 just mentioned, for after the dye injections had been discontinued for one week we noted that each macrophage contained several large irregular granules as well as many smaller ones. There is rather obviously a decrease in the staining of the tissue with the passage of time and this can be seen in gross as well as from the microscopic study. The granules do not seem to have decreased greatly in number, though many of them appear smaller. It would seem that some had grown at the expense of others or that certain of the granules had fused to form these larger masses. We are carrying out further experiments in an attempt to clear up these and other closely related points. It is felt that such data gain greatly in significance if carried on together with studies of the rate at which the dye leaves the blood stream, for these latter data give us much important information concerning the functional activities from a quantitative point of view.

Of great interest from the physiological point of view is the distribution of the dye in organs such as liver and kidneys whose activities in secreting various dye-stuffs is common knowledge. Brilliant vital red belongs to that group of dye-stuffs which passes into the urine only in small amounts. Unless very large amounts of dye are injected the small amounts of dye present in the urine are so overshadowed by the normal urinary pigments that they would not be recognized by gross inspection, but if the urine be passed several times through filter paper the latter will be found to be stained a delicate pink color. This staining is dependent upon the selective adsorption of dye by cellulose.

That the kidneys secrete so little dye is distinctly surprising in view of the fact that certain of the convoluted tubules contain such beautiful and conspicuous granules of dye. On the other hand, the epithelial cells of the liver contain no granules of brilliant vital red and yet the dye passes into the bile in considerable quantities (15-30 per cent in 24 hours). These facts support the numerous investigations of recent years which are leading to the point of view that granule formation in the epithelium of glands is a process which may be quite unrelated to the question of whether or not the gland can excrete that particular dye. Very obviously the dye can pass through the epithelial cells without forming any granules in the cytoplasm. In fact a dye may gain passage quite rapidly, so that the amount in the cytoplasm at any one time may never be enough to produce even a noticeable diffuse staining.

In general the distribution of Niagara sky blue in the body is quite similar to that described for brilliant vital red. A few minor differences are seen. From the curves given in a previous paper (14) it is evident that during the first few hours Niagara sky blue leaves the blood stream distinctly more rapidly than does brilliant vital red, though an equilibrium partition between tissues and body fluids seems to be reached rather sooner, and at a time when relatively large amounts of the dye still remain in circulation. We have repeatedly observed that animals stained with the blue dye show conspicuous staining of skin and mucous membranes many days longer than do dogs stained with similar amounts of brilliant vital red. We have no way of knowing whether this is associated with differences in elimination from the body, for unfortunately the blue dye unlike the red one is partially decolorized on admixture with bile, so that it is impossible to compare the elimination of the two in fistula dogs. The blue dye passes into the urine somewhat more readily than does the red one, but the total lost in that way is not great.

The ability of the body to decolorize many dyes is common knowledge. Some are decolorized almost immediately. Teploff (15) shows that carmine granules within the cells gradually change in shade and become yellow or black in the course of weeks, indicating slow chemical transformation. We have studied connective tissue films removed from animals at intervals following a series of injections with brilliant vital red, but we have been unable to see any change in the shade of brilliant vital red with the passage of time. We have not yet made microscopic examination of the internal organs during this stage of regression, and the possibility remains that some of the dye may undergo such change in these organs. We do know that any decolorization which does occur is not great, for it is often possible to recover 50 to 85 per cent of the dye in the bile in the course of 5 or 6 days. After that the bile may still show a reddish tint, but the amount of dye present is too small to be measured in the presence of the bile pigments. We believe that most, if not all, of the dye gradually passes from the tissues back into the blood, from which it is removed and eliminated by the liver. It is only in a few places as in the centers of the lymph nodes that the dye is retained over a period of months. Certain of these cells seem to hold to the dye with the greatest tenacity, though in time they too lose their dye.

*Experiment 25. Staining of Dog with Mixture of Brilliant Vital Red and Niagara Sky Blue.*

Dog 27-107. Female mongrel, 9.0 kg.

Injection mixture made by mixing equal parts of 1.5 per cent brilliant vital red and 1.5 per cent Niagara sky blue. Five daily intravenous injections of 14 cc. each. No dye on 6th day. Animal killed with ether on 7th. No toxicity. Tissues uniformly purple. Endocardium of heart and walls of larger vessels bluish-purple. Lungs faintly stained, and in fibrous tissue frame-work are seen irregular cells containing purple granules of dye. Many phagocytic cells in splenic pulp. They contain reddish-purple dye granules. Liver purple. Kupffer cells contain purple granules. Epithelial cells free of dye. Epithelial cells of kidney contain many dark purple dye granules. No pure red or blue granules. Macrophages in areolar tissues and in mesentery also contain only purple granules.

In conclusion, no cells anywhere contain either pure red or blue granules though all granules in spleen were reddish-purple while those in liver and kidneys were bluish-purple.

*Experiment 26. Staining of Rabbit with Mixture of Brilliant Vital Red and Niagara Sky Blue.*

Female rabbit, 3.1 kg.

Injection mixture made by mixing equal parts of 1.5 per cent Niagara sky blue and 1.5 per cent brilliant vital red. On first day 3 cc. injected. None on second day, but on following days, 5th, 6th, 7th, 7 cc. respectively were given. No toxicity. On following day animal killed with ether. Skin and mucous membrane quite purple. Spleen, liver, and kidneys also. Macrophages in connective tissue of thigh and in omentum show many discrete reddish-purple granules. Some of those in phagocytes of spleen are purplish-blue; others bluish-purple. Epithelial cells of liver contain no dye granules. Kupffer cells contain many irregular purple granules. Epithelial cells of convoluted tubules of kidney are bluish-purple.

Data of great interest are to be obtained by administering two different dyes to the same animal. It is well that they be of widely different color in order that one may recognize each in the cells. We have already shown (16) that purple plasma containing a mixture of red and blue dye may be analyzed in the spectrophotometer and the amount of each dye present in the mixture determined, and with this method it was possible to follow the elimination of each dye from the blood stream even though both dyes were present together in the plasma. It was shown (14) that each dye is finally distributed between plasma and tissues in a way characteristic of that particular dye and at a rate quite independent of the presence of the other dye in the plasma and

tissues. In certain of these physiological studies the two dyes were injected simultaneously. In others a period of intensive staining with one dye preceded the injection of the second. Histological studies of various tissues are necessary as a basis for any attempt to interpret these results.

Dog 27-107 (experiment 25) received 105 mg. of each of the two dyes daily over a period of 5 days. Gross inspection of the skin and mucous membranes showed progressively increasing purple coloration, and the animal when killed with ether showed similar changes in the internal organs. It is of interest that the aorta, and larger blood vessels generally, show a distinct excess of the bluish coloration, but there is an element of red also. This is quite in agreement with our previous observation that elastic tissue shows greater affinity for Niagara sky blue than for brilliant vital red. Microscopical examination of the aorta always confirms the gross findings and shows that the color is due to diffuse staining of the elastic fibers. Other tissues show the phagocytic reaction in a very beautiful manner. The Kupffer cells of the liver, the endothelial cells and pulp cells of the spleen, the phagocytic cells of lymph nodes and the macrophages of the connective tissues contain beautiful deposits of dyes. We were rather surprised to find that almost without exception the granules within the cells contained a mixture of the two dyes, and as a result were purple.

In one or two experiments of this sort we have found an occasional granule which appeared to be either pure red or pure blue, but this is the exception. Experiments of this sort demonstrate that these two dyes are distributed within the body in almost identically the same manner. The existence of mixed granules indicates that even within the substance of the cell the protoplasm does not discriminate between the two. Experiment 26 shows practically identical results when the two dyes are given simultaneously to a rabbit. We have other experiments with dogs and with rabbits and all of them are in agreement.

*Experiment 27. Staining of Dog with Niagara Sky Blue Followed by Staining with Brilliant Vital Red.*

Young female mongrel dog, 5.5 kg. December 16, 20 cc. 2 per cent Niagara sky blue intravenously. Similar injections on following day. December 18th and again on 19th, 20 cc. 2 per cent brilliant vital red intravenously. No toxicity. Animal killed with ether on December 20th. Skin purple. Subcutaneous con-

nective tissues bluish-purple. Lungs very slightly stained. Endocardium of heart dark purple. Aorta purple, and microscopically elastic fibers in it are diffusely stained more blue than red. Many fibers from tough subperitoneal tissue are pure blue, others bluish-purple. Spleen purple. Malpighian bodies paler and grayer than pulp. Microscopically many large irregular cells contain granules varying greatly in size and shape. Many cells contain more red dye than blue and a few contain red ones only. A few small cells contain only blue granules. Many cells contain both types but no purple granules found. Liver deep bluish-purple. Epithelial cells contain no dye granules but Kupffer cells are very large and numerous. They contain many granules of both colors but blue ones are more numerous. A very small number of cells contain only red ones and these are rather less irregular in shape than the rest. No purple granules in any of the cells. Bile deep orange color. Kidney cortex stained but medulla pale. Microscopically many granules in cells of convoluted tubules. They vary greatly in size and shape. They are red, triangular masses and granules and rods with projecting knobs, some of these knobs being of a different color than the main mass, and often granules of different color are in contact but there are no purple granules. Urine olive color, clear. Mesenteric lymph nodes deep purple. Many phagocytes contain both dyes separately but no mixed granules. Very few cells contain only one type of dye. Mesentery contains many cells with granules more or less spherical. Red and blue granules about equally numerous. In places the two types of granules seem to be loosely in contact but in no place are they fused and in no case is a zone of one dye layered about the other.

*Experiment 28. Rabbit Stained with Brilliant Vital Red Followed by Staining with Niagara Sky Blue.*

Male rabbit, 2.7 kg.

Five daily intravenous injections of 3 cc. brilliant vital red (1.5 per cent). Two days later 3 daily injections of 4 cc. each of 1.5 per cent Niagara sky blue. Animal killed with ether forty-eight hours later. Mucous membranes, kidneys, liver, spleen quite purple. In liver and spleen there is a rather less amount of both dyes than usual but red dye is more abundantly present than blue. Some Kupffer cells contain only red dye but many contain granules of each. No purple granules. No granules in epithelial cells of liver. Epithelial cells of convoluted tubules of kidney contain red and blue granules but no purple ones. In general red granules are more in evidence in the part of the cells most distant from the lumen, while in the areas bordering lumen blue granules predominate. Beautiful double segregation of dye in macrophages in connective tissues and mesentery. Very few cells contain one type of dye only. No purple granules anywhere. The blue dye is rather more in evidence in these cells than in Kupffer cells of liver and phagocytes of spleen.

A very beautiful contrast to experiments of this sort is afforded if the two dyes be injected *in successive periods*. Thus, in experiment 27 we



gave 2 daily intravenous injections of 20 cc. of 2 per cent Niagara sky blue to a dog; this was followed by 2 daily injections of brilliant vital red. The tissues, at first blue, now took on an intense purple color. At autopsy the gross appearance of the organs was identical to that when the two dyes had been given simultaneously, but the microscopical picture was surprisingly different. There was little difference as regards the diffuse staining of the elastic fibers, but we were unprepared to find that in no instance were the two dyes associated to form purple granules. Quite in contrast, the phagocytic cells scattered throughout the body contained innumerable red granules as well as innumerable pure blue granules. In almost every case these two types of granules were clearly distinct from each other though here and there granules of the two types appear to lie in contact, but neither were they fused to form mixed purple granules nor was the red dye layered about the blue dye granules as one might expect from the mode of administration. Further experiments of this sort in dogs confirmed these results. In a number of experiments on rabbits similar results were obtained. The protocol of experiment 28 is typical.

These observations raise a number of important considerations regarding the mechanism by which dye granules are formed within cells and we shall return to this subject later. We may recall here our previous inability (14) to "block" the tissues with one dye against the entrance of another. Without detailed microscopic study one might have suspected that the two dyes were taken up by two entirely separate and distinct sets of phagocytic cells, but we have just seen that such is very clearly not the case, no matter whether the dyes are given simultaneously or in sequence. It is now obvious that in the case of these two dyes the cells may contain large quantities of one dye and still fully retain their ability to take up another at the normal rate. This is all the more remarkable when we realize that these two dyes are rather closely related in their chemical properties, and with the exception of their color in most of their physical properties also. If "blockade" is ever to be obtained, one would expect it in the case of such closely similar substances which are taken up by identically the same cells and, indeed, under certain circumstances brought intimately together within the same granules. Whatever the mechanism of the segregation of dye may be, it is at least clear that

the cells react toward each dye in a way which is independent of how much of the other dye may be within the cells, or in the surrounding fluids.

In this connection we wish to emphasize our observations (14) that the amount of either dye which may be taken up is related to the concentration of that dye in the surrounding fluids. Very evidently, each dye establishes its own partition between cells and fluids independently of the presence of the other. It is tempting to compare the partition of dyes between cells and fluids to the phenomenon of partition well known to physical chemists, where a substance may be distributed between two immiscible fluids and the relative amount in each layer may be very slightly or not all influenced by the presence of other similar substances which may undergo partition according to laws peculiar to themselves. We recognize the dangers of such analogies and we do not wish to pursue the comparison further, but it is significant that in the physical as well as in the biological phenomena several substances may undergo partition largely independently of each other.

We have injected dogs with Higgins' "American" India ink and have studied the influence of this procedure upon the rate at which brilliant vital red will leave the blood stream. A report already made (17) shows that in such animals there is a very marked retention of dye in the circulating blood, and at first we had thought that the ink previously given had "blocked" the phagocytic cells or had reduced their ability to take up dye. Studies of this sort made on bile fistula dogs showed that a different explanation is much more likely, for these experiments showed that during the period of observation almost no dye is excreted into the bile if the dog had received ink at the beginning of the experiment. Control experiments showed that normally such dogs would secrete rather large quantities of dye during the four-day period of observation. The failure of the ink-treated dogs to excrete the ordinary amount of dye through the liver seemed to account quite adequately for the longer retention of the dye in the circulating blood. Under these circumstances the dye is largely retained in the body, and throughout the course of the experiment more dye was present both in plasma and in tissues. Had we remained in ignorance of this altered liver excretion we might have fallen into the error of assuming a blockade on the part of the phagocytes. It seems quite possible that others have made such mistakes when studying "blockade." Many dyes are normally eliminated by liver or kidney much more rapidly than are brilliant vital red or Niagara sky blue, and, with those dyes especially, any disturbance in the excretory power of liver or kidney would pro-

duce even more striking retention in the blood stream. Any such retention must never be credited to defective phagocytosis until all of these other factors have been given the most careful attention. We feel that experiments indicating the presence of a "blockade" should be controlled by the study of elimination through liver and through kidneys.

We have made some little progress in determining how ink inhibits the excretion of the dye by the liver, but the experiments are not yet complete. Preliminary experiments indicate that some component of the ink other than the carbon is responsible, for experiment shows that the carbon can be removed from India ink without robbing the latter of its inhibitory power. Furthermore, finely ground graphite suspensions were shown (17) to be inert. Were it not for these experiments we might conclude that the failure of the liver to excrete dye was in some way associated with the fact that the liver comes to contain large quantities of carbon, deposited largely within the Kupffer cells of the liver sinusoids.

From a somewhat more restricted point of view, a study of the distribution of ink particles in the body offers much of interest, for these details are intimately concerned with the entire problem of phagocytosis and blockade of phagocytes. The distribution of the carbon particles in the body differs from that of our vital dyes in several interesting and fundamental respects, and it seems desirable that substances so radically different from the vital dyes should be studied in conjunction with the latter in order that we may learn the physiological response of the phagocytes to each and detect and differences which exist.

For the distribution in the tissues of ink or graphite suspensions introduced into the blood stream we refer particularly to the articles of Goldmann (2, 18), Kiyono (11), Nagao (19), Foot (20), Wislocki (21), Brickner (22), Palmer and Higgins (23), Higgins and Murphy (24).

#### DISCUSSION

Most recent workers agree that there are important differences between the behavior in living tissues of "acid" and of "basic" dyes. Perhaps the difference is associated with physico-chemical reactions involving the positively charged colored ion of "basic" dyes and the negatively charged colored ion of "acid" dyes. Möllendorff (6) and Herzfeld (25) have stressed the idea that the "basic" dyes unite with electro-positive structures normally existent in many cells. They have also stressed the view that the "acid" dyes do not unite with preexisting structures within the cell, but that their storage is a process closely related to the phagocytosis of larger particles, if not identical with it. It is common knowledge that these two classes of dyestuffs may enter the same individual cells, but in general the distribution in the body and the details of staining are profoundly

different. We draw attention to these facts lest our experiments with the "acid" dyes, brilliant vital red and Niagara sky blue, be confused with known facts obtained with basic dyes.

We do not propose to discuss the many differences which exist between the various "acid" dyes.

Special reference is made to the comparative studies of Schulemann (3, 26). He and other workers have reported experiments involving double staining with acid dyes. It would lead too far to discuss in any detail the confused literature dealing with the subject. We may refer to Steckelmacher (27), Möllendorff (6), Aschoff (7), and Börner-Patzelt, Gödel, and Standenath (8) for literature. No really systematic study of the subject has been undertaken and it is no wonder that confusion prevails when the literature consists largely of isolated observations with the widest variation in dye, in species of animal and in mode of administration. Among the best controlled experiments on this subject are those of Steckelmacher (27). With the aid of lithium carmine and toluidine blue he observed double staining in liver and kidneys. His work on the frog and ours on mammals would seem to indicate that the mode of administration is of the greatest importance in the formation of mixed granules. This point of view was recognized by Schulemann (3) in certain of his earlier studies with trypan blue and vital new red. Separate injection of these two dyes gave him separate blue and red granules side by side within the cell. He encountered difficulty in producing purple granules when the two dyes were injected simultaneously, but he pointed out that vital new red is much more diffusible than trypan blue and that in all likelihood most of it is already anchored within the cells before much of the blue dye has had time to enter the cells. The result is as though the two dyes had been injected at separate intervals, and for this reason Schulemann was not surprised at his failure to find purple granules. Unfortunately he did not test out two dyes which were more nearly alike in regard to speed of penetration.

Our experiments as well as those of Schulemann and Steckelmacher all represent a rather acute type of staining. In no case did the process of staining extend over a period longer than a few days. In our own case this was felt desirable in order that the experiments might serve as controls on our previous measurements (12, 13) of the rate at which these dyes are taken up from the circulating blood.

Evans and Scott (9) report studies on vital staining in which the dyes were administered over a period of several weeks. They were particularly interested in the crystals of trypan blue which may form in the cytoplasm in these chronic types of experiments. They report finding separate deposits of trypan blue and T 148 even when the two dyes were given simultaneously, and when given

separately they found purplish granules as well as red and blue ones. The influence of the longer administration should be studied to compare the results with experiments like ours and Steckelmacher's. Admittedly the two dyes used by Evans and Scott differ considerably from each other and this must be kept in mind when attempting to correlate their results with ours.

The method of double dye injection should be of great service as an aid in the study of just how dye granules are built up within the cell. The difference in color makes it possible to follow the sequence of events. Our own experiments suggest that granules may be built up in several ways—either a few at a time, or many simultaneously and at nearly equal rate. Many more data must be secured before we can assign proper importance to the various factors which determine these finer cytological details. It should be evident that such information should be developed as a basis for physiological concepts and for physiological experiments on distribution of dye between tissues and surrounding fluids.

The experiments of a previous paper (12) showed that when large amounts of dye are given all at once or over a period of several days there is a well marked delay in the rate at which the dye leaves the blood stream. It was shown that *a priori* this may be due to a sluggish response on the part of dye-laden phagocytes, or to inefficient excretion of dye into the bile. Actual experimental study of these two possibilities showed that there is undoubtedly defective liver excretion when large doses of dye are given to the animal. In the course of 24 hours only 10 or 15 per cent of the dye may reach the bile, in contrast to 25–35 per cent when smaller doses are given. We expressed our belief that the longer retention in the blood stream may be due to this defective liver excretion alone, and not to faulty phagocytic power on the part of the tissues. If this is so we must accept the view that in the range of dosage studied, the distribution ratio of dye between plasma and tissues is constant, regardless of the dose employed. We may well be surprised at such a conclusion. It would seem to imply a vast reserve of phagocytic power not previously suspected. If we choose to believe that the tissues adapt themselves to the increased burden of larger dosage, we must also admit that this adaptation is almost instantaneous, for there seems to be no lag in the response when large doses of dye are injected or when a second dye is given simultaneously with the first (12).

Our experiments just outlined suggest a number of methods by which the tissues may adapt themselves. The increase in the number of phagocytes is one which we have observed, and this is commonly cited by others. We may also point out that increase in the size of the cell may help it to accommodate more dye without any loss of efficiency whatever. We may also call attention to the fact that with large dosage new types of cells, such as the fibroblast, take up part of the dye. This spread of the process of phagocytosis to an ever-widening group of cells may be of no little importance in maintaining the powers of the tissues to take up the large quantities of dye forced upon them. The existence of such compensating processes may cause less surprise than the fact that the process of compensation seems to be so prompt, with the result that we cannot even momentarily cause embarrassment to the phagocytic cells. We do not profess to know the relative importance of these various compensating mechanisms in relation to each other; nor may we overlook the possibility that there may be other important ways of maintaining the efficiency of this group of cells.

It may well be that methods may yet be devised to overload these cells. It may even be that refinements in the measurement of liver excretion will show that defective liver excretion will not account for quite all of the retention of dye in the plasma; but even so we cannot possibly deny the fact that the phagocytes have enormous reserve power available, or that they can adapt themselves almost perfectly to added labor within very wide limits.

The data already reported (12, 13) have given us a quantitative concept of the rate at which the body phagocytes will remove certain dyes from the circulating lymph and plasma. Those experiments on healthy living animals furnish information concerning the sum total response of all these cells combined. Details concerning the individual cells and concerning separate groups and classes of these cells are to be obtained by histological study. The attempt to combine the anatomical with the physiological approach has proved most stimulating. The results at present available represent but a beginning. Many data must still be gathered before we can know more than the elementary anatomical facts concerning the building up of dye granules within the cell. In certain instances it would seem that from the very

first the dye is deposited in a vast number of small foci within the cell, and that these tiny granules grow in size as more dye reaches the cell. In other cases it would appear that relatively few foci are active at any one time and that when the granules reach a certain size, new foci of deposition are formed within the cell. The results obtained when two dyes of different colors are given simultaneously or in sequence throw light upon these points.

Intimately connected with the theory of this granular dye deposition is the fact that as the dye deposits increase, the cell may enlarge so much that there may be no more crowding of the granules than in the earlier stages of staining when the cell is smaller and contains fewer granules. Yet, in other cases actual crowding undoubtedly does occur and with certain dyes the granules may agglutinate and needle-like crystals may even form. We believe that differences in dye employed and differences in administration account for many of these discrepancies to be found in the literature. A few of these variations are analyzed in the experiments here presented. All these anatomical details throw light upon our studies of the rate at which the dyes pass out of the plasma into the tissues. Very obviously it is desirable that we know whether the disappearance rate of dye from plasma is associated with increase in size and number of the phagocytic cells.

Our previous experiments have shown that a given dye passes from the surrounding fluids into the cell until a certain equilibrium point is reached. Dye accumulates within the cell and we find that the phagocytic activity comes almost to a standstill long before the fluids have given up all of their color. Control experiments show that dye-free phagocytes will function quite readily in the presence of plasma containing similar amounts of dye. We found also that these phagocytes may contain large amounts of this dyestuff and yet they can take up another closely related dyestuff in quite a normal manner. The histological details regarding the distribution of these two dyes form an interesting and important supplement to those observations.

#### SUMMARY

Vital staining reactions of brilliant vital red and Niagara sky blue are studied in dogs and in rabbits. Either dye alone is taken up to form red or blue granules within the cytoplasm of macrophages and of certain other cell types.

When the two dyes are injected simultaneously into the blood stream one finds that these cells build up granules which are purple from admixture of the two dyes. When several daily injections of one dye are followed by several daily injections of the other, one finds blue granules and red granules side by side within the cells, but no purple granules are found. This is thought to indicate that the dye is deposited in small foci which are active in a rather transitory way, and that the color of the granule is determined during its formative stage by the type of dye present in the fluids about the cell.

The enlargement of phagocytic cells and the increase in their number with large dosage, or with repeated offerings of the dye, represents a method by which the cells maintain their phagocytic powers at the normal level. Evidence is offered to indicate that these and perhaps other compensatory changes may take place with great rapidity, so that it has been impossible to "block" or even reduce noticeably the ability of these cells to take up additional quantities of dye.

Certain pitfalls in the experimental study of "blockade" are pointed out.

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# CONCERNING THE SPECIFICITY AND NATURE OF THE PHENOMENON OF LOCAL SKIN REACTIVITY TO VAR- IOUS BACTERIAL FILTRATES

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In previous communications (1-10) there was described a new phenomenon of local skin reactivity to culture filtrates of various microorganisms. The reactivity was induced by the injection of a filtrate into the skin of a rabbit. If 24 hours later an intravenous injection of the same filtrate was given to the same rabbit, there appeared an extremely severe hemorrhagic necrosis at the site of the previous injection. The factors determining the local skin reactivity were termed "skin preparatory factors" and those responsible for the local injury following the intravenous injection were called "reacting factors." The object of this paper is to consider the results previously published in the light of certain new data bearing upon the specificity and the nature of this phenomenon.

## *Effect of Combining Skin Preparatory Factors of One Microorganism and the Reacting Factors of Another Unrelated Microorganism*

In early papers (1, 2) a series of experiments were reported in which attempts were made to determine whether the local skin reactivity to *B. typhosus* culture filtrate could be also elicited by culture filtrates of microorganisms other than *B. typhosus*. It was shown that culture filtrates of *Streptococcus pyogenes hemolyticus* and *Streptococcus erysipelatis* failed to substitute for the *B. typhosus* culture filtrate in the phenomenon of local skin reactivity to *B. typhosus*. When, in further studies, attempts were made to elicit this phenomenon by both skin and intravenous injections of filtrates of microorganisms other than *B. typhosus* it was found that a large group of bacteria including the two above mentioned strains of streptococcus failed to elicit it. This showed that the filtrates of certain organisms lacked either skin preparatory or reacting factors or both.

Returning now to the earlier reported experiments with streptococci in which filtrates from these organisms failed to prepare for *B. typhosus* reacting factors the question arose as to whether their inability in the part was due to the lack of non-specific factors or to the lack of specific factors (in this case, *B. typhosus*).

Experiments reported below were made in order to determine whether a culture filtrate of one microörganism could prepare the skin for the injurious effect of another unrelated microörganism, provided the filtrates of both microörganisms were shown to contain potent factors.

*Methods.*—The mode of injecting and the recording of results were the same as described in previous publications (2, 4, 9). "A<sub>10</sub>," "A<sub>11</sub>" and "587" were meningococcus, *B. typhosus* ("T<sub>1</sub>" strain) and *B. coli* "agar washings" filtrates, respectively. The technique for preparation of these filtrates has been previously described (6, 7).

Anti-typhoid horse Sera H 4/68 and H/88 were recent bleedings from Horse 4. The mode of immunization of this horse was described in a recent paper (9). Anti-coli horse Serum H/86 was prepared in a similar manner.

*Experiment.*—Twelve rabbits received each three simultaneous intradermal injections of filtrates of certain microörganisms. The upper right areas of the skin of the abdomen of each of these rabbits were injected with 0.25 cc. of meningococcus filtrate ("A<sub>10</sub>"), the lower right areas with 0.25 cc. of *B. typhosus* filtrate ("A<sub>11</sub>") and the upper left areas with 0.25 cc. of *B. coli* filtrate ("587"). Twenty-four hours later the rabbits were divided into three groups of four. Each group received intravenously a different filtrate. The rabbits of the first group received per kilo of body weight 1.5 cc. of "A<sub>10</sub>" previously diluted 1:5, the second group received 1.5 cc. of "A<sub>11</sub>" previously diluted 1:5 and the third group received 1.5 cc. of "587" previously diluted 1:5. Four hours later there were found three dead rabbits in the first group. The nine surviving rabbits showed severe hemorrhagic necroses ranging in size from 2 x 2.5 to 4 x 4 cm. in all the three prepared areas.

As is seen from this experiment, skin preparatory and reacting factors of various biologically and serologically unrelated microörganisms (*i.e.*, *B. typhosus*, meningococcus, *B. coli*) are able to substitute for one another provided they have the power of eliciting the phenomenon for themselves. Apparently, therefore, filtrates from a variety of cultures are able to induce a state of reactivity in a given area of tissue. Once this state is attained the area becomes vulnerable to potent reacting factors irrespective of the microörganisms from which they are obtained.

#### *Specificity of the Skin Preparatory and Reacting Factors of Various Microörganisms*

The object of the work reported in this part of the paper was to determine whether the skin preparatory and reacting factors derived

from various microorganisms differed from each other in their antigenic structure. A series of experiments made with this object in view have been already reported in previous papers (3, 4, 5, 9, 10).

It was found that the *B. typhosus* skin preparatory factors were specifically neutralized by homologous sera. Heterologous sera of a variety of microorganisms, as well as normal sera lacking in spontaneous agglutinins for *B. typhosus*, failed to neutralize these factors. Paratyphoid A and B sera showed some neutralization. Skin preparatory factors derived from microorganisms other than *B. typhosus* were also neutralized specifically by homologous sera.

The *B. typhosus* reacting factors were also neutralized only by homologous sera.\* Normal sera free of *B. typhosus* spontaneous agglutinins as well as meningococcus therapeutic sera and certain anti-streptococcus sera failed to neutralize these factors. Meningococcus reacting factors were also neutralized specifically by homologous sera. Again, anti-typhoid immune sera showing a high neutralizing titer for *B. typhosus* reacting factors had no effect upon the meningococcus reacting factors.

It appears, therefore, from what has been stated thus far, that one substance antigenically totally different from another is able to induce a state of reactivity to it. The following experiment affords additional evidence to support this point:

*The Effect of Neutralized Reacting Factors upon Areas Prepared with Heterologous Skin Preparatory Factors.*—In this experiment there were employed many multiples of the minimal doses of reacting factors of the various bacterial filtrates necessary to elicit reactions in skin sites prepared with given amounts of each of these filtrates. The amounts of sera used were such as were found in previous experiments to give consistent neutralization with these multiples of homologous reacting factors. The experiment was carried out as follows:

*Experiment.*—Sixteen rabbits each received three simultaneous intradermal injections of various filtrates. The upper right area of the skin of the abdomen of each rabbit was injected with 0.25 cc. of undiluted "A<sub>11</sub>," the lower right areas were injected with 0.25 cc. of undiluted "A<sub>10</sub>," and the upper left areas were injected with 0.25 cc. of undiluted "587" filtrate. Twenty-four hours later the rabbits were divided into four groups of four. Each rabbit received a single intravenous injection. The rabbits of the first group received per kilo of body weight 0.5 cc. of "A<sub>11</sub>" previously diluted 1:2; the second group received 0.5 cc. of

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\* Sera of the paratyphoid group were not tested.

no visible effect upon the skin sites injected with the various non-bacterial substances.

B. Another experiment was done in which rabbits were injected intradermally with sodium arsenate, India ink and normal horse serum in various dilutions as above. Four hours after the rabbits were injected intravenously with "A<sub>11</sub>" (1 cc. of "A<sub>11</sub>" diluted 1:20, per kilo of body weight). No skin reactions followed the intravenous injection. Evidently, therefore, shortening the "incubation period" failed to produce local skin reactivity to *B. typhosus*.

As is seen from these experiments, a variety of non-bacterial substances which are able to increase the permeability of capillaries, elicit inflammation and "block" the reticulo-endothelial cells, failed to induce the state of local skin reactivity to *B. typhosus* reacting factors.

### *Attempts to Reproduce the Phenomenon by Means of Non-Bacterial Substances*

*Experiment.*—A. Six rabbits received each four simultaneous intradermal injections of crystalline egg albumin in various concentrations. The upper right areas of the skin of the abdomen were injected with 0.25 cc. of 10 per cent solution, the lower right areas with 0.25 cc. of 5 per cent solution, the upper left areas with 0.25 cc. of 2 per cent solution and the lower left areas with 0.25 cc. of 1 per cent solution. Twenty-four hours later each of the rabbits received a single intravenous injection of 1 cc. of 10 per cent solution of crystalline egg albumin per kilo of body weight. No reactions followed the intravenous injections.

B. This experiment was similar, except that normal horse serum instead of crystalline egg albumin was used both for skin and intravenous injections. The skin sites of each rabbit were prepared by injection of 0.25 cc. of the serum diluted 1:2, 1:8, 1:16, and 1:32, respectively. Three cc. of undiluted serum per kilo of body weight was injected intravenously 24 hours later. No skin reactions were obtained after the intravenous injections:

As is seen from these experiments non-bacterial protein substances, *i.e.*, normal horse serum and crystalline egg albumin, failed to reproduce the phenomenon of local skin reactivity.

### *Attempts to Transfer Passively the Local Skin Reactivity to B. Typhosus Culture Filtrates*

In these experiments attempts to transfer passively the local skin reactivity were made by using various batches of homologous immune sera derived from various animals (goats, horses and rabbits). Undiluted immune *B. typhosus* sera, and various dilutions of these sera

(1:10, 1:100 and 1:1000) were injected intradermally. Single intravenous injections of potent *B. typhosus* culture filtrates were given either 2, 5, or 24 hours after the skin injections. Since no skin reactions followed the intravenous injections, it was concluded that passive transfer of the local skin reactivity of this phenomenon cannot be realized by the employment of immune sera.

### *Various Conditions Influencing the Potency of Bacterial Culture Filtrates*

This part of the paper embodies a summary of results of a large number of experiments with various culture filtrates. Some of the results have been previously reported.

The skin preparatory and reacting factors fluctuate in potency in the same filtrate. On several occasions there was observed a gradual decrease in the potency as well as complete inactivation of a filtrate stored in the refrigerator for several months. One batch of meningococcus culture filtrate was tested at frequent intervals for a period of 4 months (10). The potency of the filtrate became somewhat lower 4 weeks after its preparation, remained unchanged for 3 months thereafter and suddenly lost its potency altogether 4 months after its preparation. It appears that the meningococcus culture filtrates are more likely to lose their potency than the *B. typhosus* filtrates, although similar difficulties were occasionally experienced with the latter.

On the other hand, some filtrates retain their potency unchanged for a considerable length of time. A filtrate of *B. Shiga* retested more than 14 months after its preparation retained its original strength.

The mode of preparation influences markedly the potency of filtrates, especially of some bacteria. As demonstrated before (6, 7), filtered washings of 24-hour old cultures of *B. typhosus* and meningococcus on solid media\* were of greater potency than any fluid culture filtrates thus far obtained. In the case of meningococcus, considerable difficulties are encountered if one attempts to obtain these factors in fluid media. Under apparently the same conditions of preparation, namely,

\* Similarly prepared *B. typhosus* filtrates were used by Ferry and Fischer for preparation of agglutinating sera and shown by them to be of superior antigenicity when compared with fluid cultures of the same bacterium (11).

with the same strain of meningococcus, with the same batch of medium of the same pH and the same length of incubation, several cultures made in the course of 1 to 2 weeks may yield filtrates of varying potency and sometimes totally inactive filtrates as well.

The abundance of growth alone is not necessarily an indication of the potency of the filtrates. This observation was made with meningococcus. Enriching substances, such as ascitic fluid, blood and glucose may be inhibitory to the production of the necessary factors. Sometimes cultures very rich in growth were found impotent, while some batches of cultures in plain broth with a comparatively poor growth were quite active.

The meningococcus factors difficult to obtain in fluid cultures are of high potency in washings of young cultures on solid media. *B. typhosus* factors can also be best obtained under these conditions. A *B. typhosus* culture filtrate (on hand at the present moment) prepared by the "agar washings" method is at least fifty times stronger than any fluid culture filtrate of the same strain thus far obtained. It is apparent, therefore, that the production of the skin preparatory and reacting factors is independent of bacterial cell autolysis (6, 7).

Different strains of the same microörganism may vary in their ability to produce these factors.

It was frequently observed that the ability of a given filtrate to kill rabbits was parallel to its skin preparatory and reacting potency. Totally inactive filtrates had no lethal effect even in intravenous doses as large as 3 cc. per kilo of body weight (meningococcus). Very potent "agar washings" filtrates of meningococcus and *B. typhosus*, on the other hand, were able to kill a small percentage of rabbits with as little as 0.005 cc. per kilo of body weight, when injected intravenously. A sudden loss in potency of such a filtrate was also accompanied by a total loss of the lethal effect.

Stock strains of certain microörganisms where filtrates were capable of consistently eliciting the phenomenon (some strains of streptococcus and pneumococcus), after having been kept in the laboratory collection for a considerable length of time, lost their power of producing the skin preparatory and reacting factors. Repeated attempts with these strains yielded only occasionally potent filtrates.

*Attempts to Reproduce the Phenomenon of Local Reactivity to Bacterial Culture Filtrates in Various Organs*

It seems to be in place to mention here that some preliminary experiments showed the possibility of reproducing this phenomenon in organs other than the skin. Rabbits were used for these experiments. The preparatory injections of bacterial filtrates were made into the renal arteries and intratracheally, respectively, in different rabbits. Twenty-four hours later the filtrates were injected intravenously. Definite lesions resulted in previously prepared organs (*i.e.*, kidney and lungs). This work will constitute the subject of a separate report.

DISCUSSION

The work reported in this paper was done in order to analyze the essential mechanism of local skin reactivity.

The first question which comes up is whether the phenomenon is a manifestation of anaphylaxis. It might be assumed that the skin injection of an antigen brings about local production of antibodies, the area becoming sensitized to a second injection of antigen which enters into combination with the antibodies present in the sensitized area.

There are several important facts which, together, sharply differentiate the phenomenon from the known phenomena of local anaphylaxis. Moreover, as will be seen later, the new data summarized and brought out by the present paper make altogether untenable the hypothesis of the anaphylactic nature of this phenomenon.

The points differentiating the phenomenon were emphasized in previous publications. In brief they are as follows: 1.—The incubation period is of extremely short duration. 2.—The reactivity disappears completely in 48 hours. 3.—The reactivity is induced by a single injection. 4.—The reaction is severe and appears rapidly (1 hour after the intravenous injections it is already possible to distinguish with the naked eye a well defined hemorrhagic necrosis). 5.—The second injection must be made by the intravenous route. All attempts to reproduce the phenomenon by repeated skin injections have failed thus far.\* 6.—The phe-

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\* It is also possible with varying degrees of success to elicit reactions in previously prepared skin sites when the second injection is given intraperitoneally (12).



nomenon cannot be reproduced by non-bacterial substances (*i.e.*, egg albumin, horse serum). 7.—The factors responsible for the phenomenon are only found in certain bacterial cultures and they vary in potency. 8.—The factors are not products of autolysis since conditions of preparation under which very little cell destruction can be expected yield highly potent filtrates. 9.—The skin preparatory and reacting factors are neutralized specifically by immune sera.

These data when compared with well known features of bacterial anaphylaxis and Arthus phenomenon put this phenomenon into an entirely new category.

Once the differentiation is made, it is also possible to show that the essential mechanism of the phenomenon does not depend on the sensitization in the presently accepted view of "anaphylaxis." In fact, if the local preparation consisted of a local production of antibodies, it would be reasonable to expect that the phenomenon would be elicited only with antigens with which these antibodies are able to enter into combination. But, as shown in the present paper, this is not the case. For, on the one hand, the skin preparatory factors of one bacterium prepare the tissue for the factors of another bacterium, even though unrelated. On the other hand, the antibodies against preparatory factors of one bacterium are not able to enter into combination with the reacting factors of another, as demonstrated by the antigenic specificity of the factors derived from various bacteria.

In view of all these considerations the phenomenon seems to be best explained on the basis of a different hypothesis. This can be formulated as follows:

The preparatory factors are endowed with the power of inducing a state of susceptibility or vulnerability in a given area of tissue (*i.e.*, skin and apparently other organs) to substances which have *primary toxicity* but which are not able to attack the tissue under the conditions of natural resistance. The skin preparatory effect is not in the nature of mere trauma, is not due to the local blockade of reticulo-endothelial cells, is not due merely to increased permeability of the capillaries or to inflammation. The vulnerability is probably due to some functional disturbance in the cells which requires a short incubation period for its appearance and which disappears rapidly. For some unknown reason the cells in this state are susceptible to severe and rapidly progressing damage provided the toxic factors are present in the general circulation.

The preparatory factors determining this state as well as the reacting factors responsible for the injury in the susceptible tissue have special characteristic features, as follows:

Both sorts of factors can be accurately titrated; they are filterable and are derived from certain bacterial cultures only; they may partially lose their potency or disappear altogether from a filtrate once highly potent; they are best obtained from young cultures under conditions of insignificant bacterial cell autolysis; their potency does not depend only on the amount of bacterial cells present in a culture; it varies with different strains of the same microorganism and a strain may cease to yield potent factors.

The potency of the factors in a given filtrate seems to parallel its lethal effect upon rabbits.

Hydrogen ion concentrations in the range from 9.0 to 4.0 have no effect upon them. The *B. typhosus* skin preparatory factors show considerable heat resistance (2).

A most interesting and important fact is that the factors determining the state of vulnerability as well as the injury producing factors can be neutralized specifically by immune sera in multiple proportions. Taken together, the properties of the factors suggest very strongly that they are identical with or closely related to true neutralizable toxins. On this assumption one may suppose the phenomenon of local skin reactivity to be the expression of a state of induced susceptibility to neutralizable toxins from microorganisms the great majority of which have not hitherto been shown to produce such toxins (paratyphoid, typhoid, coli, dysentery group, hemorrhagic septicaemia, meningococcus, a few strains of streptococci and pneumococci\*).

The results here reported also show the synergistic effect of toxins derived from various bacteria. It has been demonstrated that the induced susceptibility to a toxin of one bacterium can make a tissue receptive to the injurious effect of a large group of apparently biologically unrelated toxins. It is believed that investigations on the effect of these toxins upon different organs and studies on the synergism of

\* This phenomenon was also reproduced recently with culture filtrates of *B. pertussis* by Dr. Louis Gross and with culture filtrates of *B. influenzae* by Dr. I. A. Frisch and the author of this paper.

toxins of apparently unrelated microorganisms may elucidate the underlying mechanism of certain pathological conditions (*i.e.*, focal infections resulting in injury to distant organs, complications of infectious diseases produced by unrelated factors, etc.).

#### CONCLUSIONS AND SUMMARY

The specificity and the nature of the phenomenon of local skin reactivity to various microorganisms have been studied. It has been shown that the skin preparatory and reacting factors of various biologically and serologically unrelated microorganisms are able to substitute for each other, provided they have the power of eliciting the phenomenon for themselves.

Additional evidence has been brought concerning the antigenic specificity of the factors eliciting the phenomenon. A variety of non-bacterial substances which are able to increase the permeability of capillaries, elicit inflammation and "block" the reticulo-endothelial cells, failed to induce the state of local skin reactivity to *B. typhosus* culture filtrate. Non-bacterial protein substances (crystalline egg albumin and normal horse serum) failed to reproduce the phenomenon.

It was not possible to obtain passive transfer of the local skin reactivity.

Various conditions influencing the potency of the bacterial culture filtrates have been pointed out.

The essential nature of the phenomenon has been discussed together with its significance in relationship to disease.

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# ORAL IMMUNIZATION AGAINST THE PNEUMOCOCCUS

## USE OF BILE SALT DISSOLVED ORGANISMS, ETC., TIME OF APPEARANCE OF IMMUNITY AND DOSAGE

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In previous communications (1, 2, 3) it has been shown that white rats can be protected against multiple fatal doses of virulent pneumococci by feeding them (a) tissues of animals killed by the pneumococcus, (b) living pneumococci, and (c) acid killed cells.

The administration of the centrifuged acid killed organisms, either suspended in milk, or desiccated, was no less effective than the feeding of the infected tissue or the living bacteria, conferring on the animals a resistance to 1000 to 10,000 fatal doses. When the cells were centrifuged and heated at 80° for 2 hours, the antigenic property was considerably destroyed. The experiments seemed to show that the ingestion of the growth from 50 cc. per day per rat for a period of 10 to 15 days gave more uniform protection than smaller quantities fed over the same length of time. The duration of the active immunity to Type 1, obtained as a result of feeding for 13 days the tissues of animals killed by the same organism, was at least 4 months, at the end of which time it seemed to be as high as directly after the feeding. An insufficient number of animals prevented tests being made beyond this point. Examination of the sera of orally immunized animals (4) failed to demonstrate the presence of agglutinins or precipitins. Protective antibodies appeared in the sera of animals fed either the infected tissue or the acid killed organisms, seemingly in greater degree in the the former. They could not, however, be detected in the sera of all of the latter examined, and when found their concentration was comparatively low.

The experiments which are being reported here were done to study the effect of feeding (a) the pneumococcus grown in milk and killed by heat, (b) the degraded avirulent pneumococcus (c) the mechanic-

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ally disrupted cell, (d) the bile salt dissolved cell in varying amounts. There are also recorded the results of experiments in which were studied (e) the number of feedings required to produce an immunity, (f) the time at which protection first appears, (g) whether treated animals can resist subcutaneous as well as intraperitoneal injection, and (h) the effect of feeding much smaller quantities of acid killed pneumococci than have been used heretofore. All the experiments were done with Type 1, using white rats, and injecting the test dose in a volume of 0.20 cc. intraperitoneally, except in measuring subcutaneous resistance in (g) above. Except in the experiment in which desiccated material was employed, the organisms were grown daily.

The attempt has been made in the present work to find the form of material, which when administered by mouth, would yield the greatest degree of protection most promptly with the minimum number of feedings and the minimum size of the dose. No emphasis has been placed on the duration of the immunity although figures are given in the tables which show the intervals between the last feeding and the days of the test. We are trying now to learn what determines the length of time during which the animal possesses the increased resistance. It seems at present that the number of feedings plays a part.

*Feeding the Pneumococcus Grown in Sterile Milk and Killed by Heat.—*

The sterile milk was inoculated with broth cultures and incubated at 37° for about 20 hours. The culture was killed by heating in a water bath for 1 hour at 60°C. It was then placed in the cage with the other food.

Two experiments were done. The results of the first are given in Table I and show that the pneumococcus, when fed in this form, in amounts equivalent to 3 cc. per rat per day, will produce an increased resistance to the virulent organism.

The extent of the protection is not so great as was obtained when larger quantities of the acid killed organisms were fed, either freshly centrifuged and resuspended in milk, or desiccated and mixed with moistened cracker meal. Accurate comparison with the effect of feeding the growth from equal volumes of broth grown organisms obtained in our earlier experiments, is somewhat difficult to make, because in those experiments the treated animals were tested with excessively large doses, and most of them succumbed. Later in the present paper additional data are given with regard to the ingestion of acid killed bacteria from 1 cc. and 5 cc. growth in broth.

The number of days on which the bacteria were fed varied between 11 and 17, but this caused no detectable difference in the degree of protection produced. The rats used in a second experiment were considerably larger than those comprising Table I and gave less uniform results. This difference in response to the same treatment is probably not owing to a difference in the age of the animals since it was not observed where acid killed pneumococci were fed (3). Heating the cultured milk at 60°C. for 1 hour apparently did not destroy the constituent of the cell responsible for the production of the immunity.

TABLE I

*Immunizing Effect of Feeding Heat Killed Pneumococcus Milk Culture*

C = Control; E = Treated rat; D = Died, days; S = Survived

Wt.	Dose	Result	No. of days fed	*Interval	Wt.	Dose	Result	No. of days fed	*Interval	Wt.	Dose	Result	No. of days fed	*Interval
gm.	cc.				gm.	cc.				gm.	cc.			
*C158	10 <sup>-7</sup>	D2			E154	10 <sup>-6</sup>	D3	15	1	E273	10 <sup>-5</sup>	D2	14	21
C165	10 <sup>-6</sup>	D2			E171	10 <sup>-5</sup>	S.	15	1					
E158	10 <sup>-7</sup>	S	15	1						*C213	10 <sup>-8</sup>	S		
E160	10 <sup>-6</sup>	S	15	1	*C168	10 <sup>-8</sup>	S			C225	10 <sup>-7</sup>	D3		
					C170	10 <sup>-7</sup>	D4			C227	10 <sup>-6</sup>	D2		
*C138	10 <sup>-8</sup>	D2			C176	10 <sup>-6</sup>	D2			E195	10 <sup>-6</sup>	S	11	22
C148	10 <sup>-7</sup>	D2			E144	10 <sup>-6</sup>	S	16	1	E203	10 <sup>-5</sup>	S	11	22
C149	10 <sup>-6</sup>	D3			E185	10 <sup>-5</sup>	S	16	1	E213	10 <sup>-5</sup>	D2	11	22
E134	10 <sup>-7</sup>	S	17	1	E188	10 <sup>-5</sup>	S	16	1					
E144	10 <sup>-6</sup>	S	17	1						*C233	10 <sup>-7</sup>	D2		
					*C206	10 <sup>-8</sup>	D2			C241	10 <sup>-6</sup>	D3		
*C155	10 <sup>-8</sup>	D3			C229	10 <sup>-7</sup>	D2			E194	10 <sup>-5</sup>	D3	11	28
C161	10 <sup>-7</sup>	D3			C237	10 <sup>-6</sup>	D2			E214	10 <sup>-5</sup>	D2	11	28
C169	10 <sup>-6</sup>	D3			E191	10 <sup>-6</sup>	D3	11	21	E227	10 <sup>-5</sup>	S	11	28
E145	10 <sup>-7</sup>	S	15	1	E213	10 <sup>-5</sup>	D2	11	21					

\* A new day.

The average amount ingested was 3 cc. per day for the number of days indicated.

° Elapsed number of days between last feeding and test.

*Feeding the Degraded\* Avirulent Pneumococcus.*—This strain of Type 1 pneumococcus was derived from the virulent strain used in the

\* In a preliminary communication (*Proc. Soc. Exp. Biol. and Med.*, 1929, 26, 565-567) this organism was referred to as "avirulent." It would seem more accurate to describe it as "degraded avirulent" in view of its properties.



other parts of this work by adding 1 volume of "N" HCl acid solution to 14 volumes of glucose meat extract broth culture, and subculturing before the growth was killed.

The organism failed to kill mice when 1 cc. of a 24 hour culture was injected intraperitoneally. It appeared at first to be entirely insoluble in sodium glyco-

TABLE II

*Effect of Feeding HCl Killed Degraded Avirulent Pneumococcus*

Wt.	Dose	Result	*In- terval	Wt.	Dose	Result	*In- terval	Wt.	Dose	Result	*In- terval
gm.	cc.			gm.	cc.			gm.	cc.		
*C70	10 <sup>-8</sup>	D2		E116	10 <sup>-8</sup>	D3	1	C104	10 <sup>-7</sup>	D2	
C74	10 <sup>-7</sup>	D1		E120	10 <sup>-7</sup>	D2	1	C110	10 <sup>-6</sup>	D2	
C77	10 <sup>-6</sup>	D2		E144	10 <sup>-6</sup>	D4	1	E93	10 <sup>-9</sup>	S	5
E61	10 <sup>-8</sup>	D2	12					C108	10 <sup>-8</sup>	S	5
E66	10 <sup>-7</sup>	D3	12	*C118	10 <sup>-8</sup>	S		E108	10 <sup>-7</sup>	D3	5
E68	10 <sup>-6</sup>	D2	12	C133	10 <sup>-7</sup>	D3					
				E127	10 <sup>-8</sup>	D2	3	*C102	10 <sup>-8</sup>	D3	
*C138	10 <sup>-8</sup>	D2		E129	10 <sup>-7</sup>	D4	3	C103	10 <sup>-7</sup>	D2	
C144	10 <sup>-7</sup>	D2		E131	10 <sup>-6</sup>	D2	3	C122	10 <sup>-7</sup>	D2	
C158	10 <sup>-6</sup>	D2						C123	10 <sup>-6</sup>	D2	
E135	10 <sup>-8</sup>	D**	14	*C105	10 <sup>-8</sup>	D5		E122	10 <sup>-8</sup>	S	8
E140	10 <sup>-7</sup>	D2	14	C109	10 <sup>-7</sup>	D2		E123	10 <sup>-8</sup>	S	8
E147	10 <sup>-6</sup>	D2	14	E148	10 <sup>-8</sup>	D2	4	E127	10 <sup>-8</sup>	S	8
				E175	10 <sup>-7</sup>	D2	4	E127	10 <sup>-7</sup>	D4	8
*C118	10 <sup>-8</sup>	D2		E185	10 <sup>-6</sup>	D1	4	E133	10 <sup>-7</sup>	D3	8
C118	10 <sup>-7</sup>	D2						E138	10 <sup>-7</sup>	D3	8
C144	10 <sup>-6</sup>	D2		*C90	10 <sup>-9</sup>	S		E146	10 <sup>-6</sup>	D2	8
				C94	10 <sup>-8</sup>	D2					

\* A new day.

\*\* Very sick; killed.

The average amount ingested was equivalent to 50 cc. growth per day, for 3 days in the case of the first 2 groups, and for 9 days in the remaining 5 groups.

° Elapsed number of days between last feeding and test.

cholate, but on adding considerably larger quantities of this salt than sufficed to dissolve the virulent strain, solution resulted. Organisms removed from blood agar and suspended in 0.90 per cent salt solution appear to dissolve somewhat more easily than broth cultures. No capsule could be demonstrated.

It was agglutinated approximately to the same extent when equal volumes (0.5 cc.) of the culture and 1-20 Type 1, 1-20 Type 2, and 1-5 Type 3 antisera were

mixed and incubated at 37°C.\* The precipitin test performed by mixing 0.5 cc. of a Berkefeld filtrate of a beef heart culture with 0.5 cc. of 1-10 Type 1, 1-10 Type 2 and 1-5 Type 3 antisera showed no precipitate after incubating for 20 hours at 37°C.

Two experiments were done with this strain. In the first, the organism was grown in glucose meat extract broth, and before use was treated with "N" HCl

TABLE III  
*Effect of Feeding Degraded Avirulent Pneumococcus*

Wt.	Dose	Result	Wt.	Dose	Result
gm.	cc.		gm.	cc.	
*C57	10 <sup>-3</sup>	S	**C60	10 <sup>-3</sup>	S
C61	10 <sup>-3</sup>	S	C61	10 <sup>-3</sup>	S
C63	10 <sup>-3</sup>	D3	C72	10 <sup>-3</sup>	D3
C68	10 <sup>-3</sup>	D2	C80	10 <sup>-3</sup>	D2
C70	10 <sup>-7</sup>	D2	C90	10 <sup>-7</sup>	D2
C79	10 <sup>-7</sup>	D2	C92	10 <sup>-7</sup>	D3
E57	10 <sup>-3</sup>	S	C91	10 <sup>-6</sup>	D2
E60	10 <sup>-3</sup>	S	C95	10 <sup>-6</sup>	D2
E64	10 <sup>-3</sup>	D2	E62	10 <sup>-3</sup>	S
E69	10 <sup>-3</sup>	S	E67	10 <sup>-3</sup>	S
E70	10 <sup>-7</sup>	S	E75	10 <sup>-3</sup>	D3
E79	10 <sup>-7</sup>	D2	E76	10 <sup>-3</sup>	D2
			E75	10 <sup>-7</sup>	D2
			E81	10 <sup>-7</sup>	D3
			E82	10 <sup>-6</sup>	D2
			E91	10 <sup>-6</sup>	D3

\* Three days after the last feeding.

\*\* Six days after the last feeding.

The average amount ingested was equivalent to 50 cc. growth per day per rat on each of 3 consecutive days. The culture, although made up to N/15 HCl and kept at room temperature for 2 hours (at which time feeding took place), was still alive.

acid solution to make a "N/15" concentration and was left at room temperature for 2 hours, at which time subcultures demonstrated that the growth had been killed. The quantity fed was equivalent to 50 cc. growth per rat per day. The

\* Similar tests done at 56°C. with Types 1 to 13 pneumococcus antisera showed about the same degree of agglutination in all. Type numbers are those used at Bureau of Laboratories, Department of Health, New York City.

first two groups of E rats listed in Table II were fed this amount on 3 successive days. The remaining 5 groups received 6 additional feedings and then the resistance of all the rats was measured. It will be seen that the fatal dose for controls is, with one exception,  $10^{-8}$  cc. The treated rats succumb to the same dose, except on 2 days when  $10^{-7}$  cc. was required to kill. On the day when  $10^{-7}$  cc. was required to kill a control,  $10^{-8}$  cc. was fatal for an E rat. At first, the survival of the E rats injected with  $10^{-8}$  cc. in the last two groups was regarded as the result of chance, especially since in one of the preceding groups a control survived  $10^{-8}$  cc. whereas an E rat succumbed to this dose. It was thought that no protection followed ingestion of this organism.

The experiment recently has been repeated with some modification. The pneumococcus was grown in beef heart broth without glucose, and 3 consecutive daily feedings were given, each equivalent to the growth of 50 cc. per rat. As in the first experiment, HCl acid was added to make N/15 concentration, but subcultures at this time showed that the culture had not been killed. It would seem that this organism was somewhat more resistant to acid than the virulent one. Table III gives the data obtained when the rats were injected with virulent Type 1. Three days after the last feeding one of the treated rats survived one fatal dose, another ten fatal doses. Six days following the last ingestion of bacteria none of the treated rats survived single fatal doses or 10 and 100 fold multiples of this dose. The survival of the other two animals suggests, when considered with the survival of some of the treated rats in Table II, that, though the protection afforded by feeding the degraded Type 1 pneumococcus is slight, it nevertheless seems to exist.

*Feeding Mechanically Disrupted Cells.*—The object of this experiment was to determine whether the intact cell is essential in this method of immunization or whether the component parts would function equally well.

The organism used was a virulent strain. The bacteria were desiccated in a partial vacuum and pulverized in a pebble mill for 18 hours. Microscopic examination failed to show any whole cells. There were, in all probability some intact organisms in this powder, but it is not likely they were sufficiently numerous to produce the high degree of immunity observed. After distributing the disrupted cells in milk, the whole was placed in the cage. The rats had been fasted 18 hours daily and the volume of milk was kept small, so that all of the mixture was quickly consumed. Feeding took place on 16 days, each rat receiving the equivalent of 50 cc. growth per day. Table IV shows the results. The treated rats survived 1000 and 10,000 fatal doses.

The protection on the whole is as great and as uniform as when the acid killed intact organism is used. It is apparent that it is not neces-

sary to feed the intact pneumococcus; the derivatives alone are sufficient. This result is confirmed by the experiments done with bile salt dissolved bacteria.

*Feeding Bile Salt Dissolved Pneumococci.*—Several experiments were done with dissolved cells in order to confirm the observation that the whole cell is not indispensable for producing an immunity by feeding. It seemed moreover that, if it should prove to be effective, the ingestion of the material in solution might yield very uniform results.

TABLE IV

*Immunizing Effect of Feeding Desiccated Mechanically Disrupted Pneumococcus*

Wt.	Dose	Result	°In- terval	Wt.	Dose	Result	°In- terval	Wt.	Dose	Result	°In- terval
gm.	cc.			gm.	cc.			gm.	cc.		
*C157	10 <sup>-8</sup>	S		E164	10 <sup>-6</sup>	S	16	E132	10 <sup>-6</sup>	S	19
C166	10 <sup>-7</sup>	D8		E170	10 <sup>-5</sup>	S	16	E133	10 <sup>-5</sup>	S	19
E152	10 <sup>-8</sup>	S	6					E142	10 <sup>-4</sup>	S	19
E168	10 <sup>-7</sup>	S	6	*C174	10 <sup>-8</sup>	S					
				C180	10 <sup>-7</sup>	D2		*C198	10 <sup>-8</sup>	D2	
*C143	10 <sup>-8</sup>	S		C211	10 <sup>-6</sup>	D2		C205	10 <sup>-7</sup>	D2	
C170	10 <sup>-7</sup>	D6		E176	10 <sup>-6</sup>	S	18	C214	10 <sup>-6</sup>	D2	
E138	10 <sup>-7</sup>	S	9	E153	10 <sup>-5</sup>	S	18	E97	10 <sup>-6</sup>	S	21
E138	10 <sup>-6</sup>	S	9	E167	10 <sup>-4</sup>	D3	18	E117	10 <sup>-6</sup>	S	21
								E131	10 <sup>-5</sup>	S	21
*C169	10 <sup>-8</sup>	S		*C173	10 <sup>-8</sup>	S		E138	10 <sup>-5</sup>	S	21
C182	10 <sup>-7</sup>	D2		C179	10 <sup>-7</sup>	D2		E159	10 <sup>-4</sup>	S	21
C187	10 <sup>-6</sup>	D2		C181	10 <sup>-6</sup>	D2		E158	10 <sup>-4</sup>	D3	21
E158	10 <sup>-6</sup>	S	16								

\* A new day.

Each rat received the equivalent of 50 cc. growth per day on each of 16 days.

° Elapsed number of days between last feeding and test.

The organism was grown in beef heart broth. The centrifuged bacteria were dissolved in sodium taurocholate. In the first two experiments of this kind, the solution was not filtered. Cracker meal was added, the whole was intimately mixed and placed in the cage. A preliminary period of fasting was employed to ensure better absorption of the active material. Each rat received the equivalent of 50 cc. growth per day for 21 days. The data of this experiment are recorded in Table V, and show that bile salt dissolved pneumococci, when administered by mouth create a consistent increased resistance against the virulent organism.

The experiment was repeated, both the quantity fed daily and the number of feedings being reduced. The data are given in Table VI. The first group of rats

TABLE V

*Immunizing Effect of Feeding Sodium Taurocholate Dissolved Pneumococcus*

Wt.	Dose	Result	*Interval	Wt.	Dose	Result	*Interval
gm.	cc.			gm.	cc.		
*C130	10 <sup>-8</sup>	D3		*C132	10 <sup>-8</sup>	D6	
C150	10 <sup>-7</sup>	D8		C134	10 <sup>-7</sup>	D6	
E194	10 <sup>-7</sup>	S	13	C137	10 <sup>-6</sup>	D2	
E199	10 <sup>-7</sup>	S	13	E174	10 <sup>-8</sup>	S	24
				E176	10 <sup>-7</sup>	S	24
*C146	10 <sup>-8</sup>	Sick but recovered		E228	10 <sup>-6</sup>	D2	24
C148	10 <sup>-7</sup>	D5					
E161	10 <sup>-7</sup>	S	14	*C160	10 <sup>-8</sup>	S	
E182	10 <sup>-6</sup>	D3	14	C175	10 <sup>-7</sup>	D2	
				C186	10 <sup>-6</sup>	D4	
*C132	10 <sup>-8</sup>	D6		E225	10 <sup>-8</sup>	S	26
C138	10 <sup>-7</sup>	D6		E227	10 <sup>-7</sup>	S	26
E192	10 <sup>-8</sup>	S	17	E254	10 <sup>-6</sup>	S	26
E198	10 <sup>-7</sup>	S	17				
E199	10 <sup>-6</sup>	S	17	*C151	10 <sup>-8</sup>	D2	
				C158	10 <sup>-7</sup>	D2	
*C135	10 <sup>-8</sup>	D2		C171	10 <sup>-6</sup>	D2	
C138	10 <sup>-7</sup>	D		E232	10 <sup>-8</sup>	S	28
C150	10 <sup>-6</sup>	D6		E233	10 <sup>-7</sup>	S	28
E199	10 <sup>-8</sup>	S	20	E247	10 <sup>-6</sup>	D2	28
E207	10 <sup>-7</sup>	S	20				
E208	10 <sup>-6</sup>	D2	20	*C157	10 <sup>-8</sup>	S	
				C166	10 <sup>-7</sup>	D8	
				**E184	10 <sup>-7</sup>	S	41
				**E200	10 <sup>-7</sup>	S	41

\* A new day.

\*\* Had litter 1 month before the test, which was 10 days after last feeding.

The treated rats received 21 feedings, each equivalent to 50 cc. growth per rat. The feedings extended over a period of 5 weeks and ceased 2 weeks before the first group in the above table was tested.

° Elapsed number of days between last feeding and test.

shown in the table was fed the equivalent of the growth from 25 cc. culture per rat on each of 3 consecutive days. Six days after the last feeding they were tested along with controls. The E rat which survived 10<sup>-6</sup> cc. illustrates that an in-

creased resistance had already been built up. The E rats comprising the second and third groups, in addition to having received the 3 feedings mentioned, were now given 1 and 2 additional feedings respectively. The animals of the remaining 4 groups were fed 6 times in all. Examination of the table shows that the ingestion of the bile salt dissolved organisms was followed by the ability to resist 10,000 fatal doses of the organism.

TABLE VI

*Immunizing Effect of Feeding Sodium Taurocholate Dissolved Pneumococcus*

Wt.	Dose	Result	*In- terval	Wt.	Dose	Result	*In- terval	Wt.	Dose	Result	*In- terval
gm.	cc.			gm.	cc.			gm.	cc.		
*C53	10 <sup>-8</sup>	S		E81	10 <sup>-7</sup>	S	1	E91	10 <sup>-4</sup>	S	3
C60	10 <sup>-7</sup>	D2		E83	10 <sup>-6</sup>	S	1				
C63	10 <sup>-6</sup>	D1		E84	10 <sup>-5</sup>	S	1	*C77	10 <sup>-8</sup>	D3	
E52	10 <sup>-8</sup>	S	6					C82	10 <sup>-7</sup>	D2	
E59	10 <sup>-7</sup>	D2**	6	*C70	10 <sup>-8</sup>	D3		C91	10 <sup>-6</sup>	D2	
E66	10 <sup>-6</sup>	S	6	C74	10 <sup>-7</sup>	D5		E106	10 <sup>-7</sup>	S	7
				C81	10 <sup>-6</sup>	D2		E111	10 <sup>-6</sup>	S	7
*C70	10 <sup>-8</sup>	D2		E71	10 <sup>-7</sup>	S	1	E115	10 <sup>-5</sup>	S	7
C74	10 <sup>-7</sup>	D1		E77	10 <sup>-6</sup>	S	1	E122	10 <sup>-4</sup>	S	7
C75	10 <sup>-6</sup>	D1		E82	10 <sup>-5</sup>	S	1				
E72	10 <sup>-8</sup>	S	3					*C90	10 <sup>-8</sup>	D2	
E73	10 <sup>-7</sup>	S	3	*C68	10 <sup>-8</sup>	D2		C94	10 <sup>-7</sup>	D2	
E80	10 <sup>-6</sup>	S	3	C77	10 <sup>-7</sup>	D2		C102	10 <sup>-6</sup>	D2	
				C79	10 <sup>-6</sup>	D1		†E89	10 <sup>-6</sup>	D4	10
*C72	10 <sup>-8</sup>	S		E82	10 <sup>-7</sup>	S	3	E100	10 <sup>-5</sup>	S	10
C75	10 <sup>-7</sup>	D2		E86	10 <sup>-6</sup>	S	3	E128	10 <sup>-4</sup>	S	10
C76	10 <sup>-6</sup>	D2		E91	10 <sup>-6</sup>	S	3	E158	10 <sup>-3</sup>	D1	10

\* A new day.

\*\* Ill before injection.

† Has an infected leg.

The E rats in the first group received 3 feedings, those in the second and third groups 4 and 5 feedings respectively, and the remaining ones 6 feedings, each equivalent to 25 cc. growth. In addition each E rat was fed 2.5 mg. sodium glycocholate.

° Elapsed number of days between last feeding and test.

In the two experiments just described, there remained the possibility that the intact cells, though few in number, might have been responsible for the immunity which was created. Hence in all the subsequent experiments, the bile dissolved material was passed through a Berkefeld filter before it was fed to the rats.

It now appeared that a portion, at least, of the increased resistance could be elicited by 3 consecutive feedings. It was then decided to determine whether 3 consecutive feedings would be followed by the maximum degree of protection, and to make the first test of the animals' resistance 24 hours after the last of 3 such feedings instead of 6 days as was the case with those rats in the preceding experiment which were fed only 3 times.

TABLE VII

*Immunizing Effect of Feeding Berkefeld Filtrate of Sodium Glycocholate Dissolved Pneumococcus*

Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
*C62	10 <sup>-8</sup>	S	*C71	10 <sup>-8</sup>	D3	*C82	10 <sup>-8</sup>	D2
C83	10 <sup>-7</sup>	D2	C72	10 <sup>-7</sup>	D2	C84	10 <sup>-7</sup>	D2
E63	10 <sup>-8</sup>	S	E55	10 <sup>-7</sup>	S	C105	10 <sup>-8</sup>	D2
E83	10 <sup>-7</sup>	S	E65	10 <sup>-6</sup>	D2	**E72	10 <sup>-7</sup>	D2
			E71	10 <sup>-5</sup>	D3	**E73	10 <sup>-6</sup>	D2
*C63	10 <sup>-8</sup>	D2				**E73	10 <sup>-6</sup>	S
C85	10 <sup>-7</sup>	D2	*C84	10 <sup>-8</sup>	S	**E80	10 <sup>-5</sup>	S
E62	10 <sup>-7</sup>	S	C87	10 <sup>-7</sup>	D2	**E114	10 <sup>-5</sup>	S
E87	10 <sup>-6</sup>	S	C103	10 <sup>-6</sup>	D2			
			E61	10 <sup>-7</sup>	D2	*C90	10 <sup>-8</sup>	D3
*C51	10 <sup>-9</sup>	S	E64	10 <sup>-7</sup>	S	C93	10 <sup>-7</sup>	D3
C70	10 <sup>-8</sup>	D2	E68	10 <sup>-6</sup>	S	C95	10 <sup>-6</sup>	D2
C72	10 <sup>-7</sup>	D2	E69	10 <sup>-6</sup>	S	**E60	10 <sup>-6</sup>	D2
E51	10 <sup>-7</sup>	S	E75	10 <sup>-5</sup>	S	**E66	10 <sup>-6</sup>	D4
E71	10 <sup>-6</sup>	S	E116	10 <sup>-5</sup>	S	**E66	10 <sup>-5</sup>	S
E73	10 <sup>-5</sup>	S				**E79	10 <sup>-5</sup>	D2

\* A new day.

\*\* These rats ate of the cracker meal-filtrate organism mixture indifferently and it is not certain that some of them ate any. Feeding of filtrate took place on the 3 days immediately preceding the day on which the first group above was injected. The remaining 6 groups were injected 4, 5, 7, 8, 10 and 12 days, respectively, after the last feeding. Average amount fed was equivalent to 30 cc. culture per rat each time, together with 5 mg. sodium glycocholate.

Each rat was fed the Berkefeld filtrate of sodium glycocholate dissolved pneumococci equivalent to 30 cc. culture per day on 3 successive days. The rats were fasted for 18 hours and the cracker meal-filtrate mixture was placed in the cage. Unfortunately some of the animals ate of it indifferently and one could not be

certain that every rat had eaten at least a portion. A rough separation into 2 groups was made, comprising those animals which had eaten liberally and those observed to have barely taken any. The results of the test appear in Table VII, and show that 3 feedings of cell free filtrate protected rats against 1000 fatal doses. At least a portion of this immunity appeared 24 hours after the last dose; all of it was present 5 days after the last dose. The irregularity in the reaction of the rats comprising the last 2 of the 7 groups is probably owing to the fact that these animals were the ones which were observed to have eaten sparingly of the mixture of filtrate and cracker meal.

TABLE VIII

*Time of Appearance of Immunity Produced in Rats by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)*

Tested 24 hrs. after the feeding			Tested 48 hrs. after the feeding		
Wt.	Dose	Result	Wt.	Dose	Result
gm.	cc.		gm.	cc.	
C74	10 <sup>-8</sup>	D2	C64	10 <sup>-8</sup>	D2
C65	10 <sup>-8</sup>	D3	C71	10 <sup>-8</sup>	D2
C79	10 <sup>-7</sup>	D2	C81	10 <sup>-7</sup>	D2
C82	10 <sup>-7</sup>	D2	C82	10 <sup>-7</sup>	D2
C85	10 <sup>-6</sup>	D2	C82	10 <sup>-6</sup>	D2
C92	10 <sup>-6</sup>	D2	C90	10 <sup>-6</sup>	D2
E71	10 <sup>-8</sup>	S	E70	10 <sup>-8</sup>	S
E78	10 <sup>-8</sup>	D3	E71	10 <sup>-8</sup>	S
E80	10 <sup>-7</sup>	S	E73	10 <sup>-7</sup>	S
E80	10 <sup>-7</sup>	D3	E80	10 <sup>-7</sup>	S
E82	10 <sup>-6</sup>	D2	E91	10 <sup>-6</sup>	S
E114	10 <sup>-5</sup>	D2	E93	10 <sup>-5</sup>	S

Each E rat received the equivalent of 50 cc. growth and 8 mg. sodium glycocholate.

This experiment definitely indicated that the presence of the whole cell was not essential in the production of an immunity by the oral route.

It was now known that animals fed the sterile filtrate on 3 consecutive days were at least partially protected 72 hours after the first feeding. This suggested the advisability of determining more exactly the degree of protection at such early stages, and of learning whether the immunity might appear sooner than 72 hours after the first ingestion, and possibly after fewer than 3 feedings.



In the next experiment (Table VIII) each rat was fed the Berkefeld filtrate of sodium glycocholate dissolved pneumococci equivalent to 50 cc. growth in a single feeding. Each rat was fed separately, to be certain that every animal received its portion. One half of the treated rats were tested 24 hours later and the other half 48 hours after the feeding. In the 24 hour test one rat survived a single fatal dose and one survived 10 such doses. Two other E rats receiving the same amounts and the remaining 4 injected with larger quantities, succumbed. Forty-eight hours after the ingestion of the bile dissolved bacteria however, all 6 treated rats, injected with quantities varying from 1 to 1000 fatal doses survived. This

TABLE IX

*Time of Appearance of Immunity Produced by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)*

Tested 24 hrs. after the feeding			Tested 48 hrs. after the feeding		
Wt.	Dose	Result	Wt.	Dose	Result
gm.	cc.		gm.	cc.	
C60	$10^{-8}$	D3	C70	$10^{-8}$	D2
C75	$10^{-8}$	D3	C80	$10^{-8}$	S
C75	$10^{-7}$	D2	C87	$10^{-7}$	D2
C78	$10^{-7}$	D2	C93	$10^{-7}$	D2
C100	$10^{-6}$	D2	C97	$10^{-6}$	D2
E85	$10^{-6}$	D3	E90	$10^{-6}$	S
E85	$10^{-6}$	D2	E99	$10^{-6}$	S
E87	$10^{-7}$	D2	E95	$10^{-7}$	S
E95	$10^{-7}$	D2	E93	$10^{-7}$	S
E95	$10^{-6}$	D3	E72	$10^{-6}$	S
E95	$10^{-6}$	D2	E112	$10^{-6}$	S

Each rat received the equivalent of 50 cc. growth and 5 mg. sodium glycocholate.

experiment was repeated, in all its details, except that the quantity of bile salt used to dissolve the cells was reduced so that each rat was given 5 mg. instead of 8. Subsequently this was even further reduced. The data appear in Table IX. This time none of the E rats survived when tested 24 hours after the single ingestion. All those injected after 48 hours survived.

It seems therefore that the increased resistance is present fairly consistently and rather marked at the end of 48 hours.

Having found that the immunity is created so promptly it was next decided to learn whether the quantity of culture fed could be reduced

from 50 to 5 cc. without delaying the time of its appearance or reducing its extent.

Table X shows that one treated rat survived an injection of 10 fatal doses 24 hours following a single feeding equivalent to 5 cc. of growth. At the end of 47 hours the treated rats were able to tolerate at least 1000 fatal doses and after 72 hours, 10,000 such doses.

It seemed worth while to study the effect of feeding still smaller amounts, and to determine whether the treated rats would survive

TABLE X

*Time of Appearance of Immunity by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)*

24 hrs. after feeding			47 hrs. after feeding			72 hrs. after feeding		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C74	$10^{-8}$	D2	C91	$10^{-8}$	D4	C106	$10^{-8}$	D3
C75	$10^{-8}$	D2	C96	$10^{-8}$	D4	C116	$10^{-7}$	D2
C79	$10^{-7}$	D2	C101	$10^{-7}$	D3	E76	$10^{-7}$	S
C84	$10^{-7}$	D2	C107	$10^{-7}$	D3	E86	$10^{-6}$	S
E70	$10^{-8}$	D2	E86	$10^{-8}$	S	E98	$10^{-5}$	S
E78	$10^{-8}$	D2	E86	$10^{-8}$	S	E107	$10^{-4}$	S
E83	$10^{-7}$	D2	E86	$10^{-7}$	S			
E84	$10^{-7}$	S	E92	$10^{-7}$	S			
			E93	$10^{-6}$	S			
			E94	$10^{-5}$	S			

Each rat received the equivalent of 5 cc. growth and 1 mg. sodium glycocholate.

subcutaneous injection of the pneumococci as well as intraperitoneal. The experiments which were done to find the answer to the latter will be described first.

A group of rats was fed the Berkefeld filtrate of sodium glycocholate dissolved pneumococci. Twenty-four hours later (Table XI) two of this group were injected subcutaneously with  $10^{-8}$  cc., and two with  $10^{-7}$  cc. Four controls were similarly injected. There was no indication of the presence of any immunity at this time. Forty-eight hours following the feeding, several additional animals were injected and the result was the same. After 72 hours the remaining rats of the group were tested. The fatal dose for the controls was between  $10^{-8}$  and  $10^{-7}$  cc. Among

the treated rats, the one injected with  $10^{-5}$  cc. died, those receiving smaller doses and the rat receiving  $10^{-4}$  cc. survived. The results of this experiment seemed to show that the resistance to a subcutaneous injection appears 1 day later than to an intraperitoneal one. The survival of the animal injected with  $10^{-4}$  cc. indicates that the degree of protection may be as high when tested by the subcutaneous route as by the intraperitoneal. A repetition of this experiment is illustrated in Table XII. Forty-eight hours after the feeding four treated rats were injected with  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-7}$  and  $10^{-6}$  cc., respectively; of these, one ( $10^{-8}$  cc.) died, and the heart's blood was found to be sterile. Of four untreated rats injected subcu-

TABLE XI

*Immunity to Subcutaneous Injection Produced by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)*

24 hrs. after feeding			48 hrs. after feeding			72 hrs. after feeding		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C77	$10^{-8}$	S	C75	$10^{-8}$	S	C81	$10^{-8}$	D5
C81	$10^{-8}$	S	C79	$10^{-8}$	S	C80	$10^{-8}$	S
C83	$10^{-7}$	S	C86	$10^{-7}$	S	C62	$10^{-7}$	D3
C88	$10^{-7}$	D4	C80	$10^{-7}$	D3	C83	$10^{-7}$	D3
E77	$10^{-8}$	S	E71	$10^{-8}$	S	C93	$10^{-7}$	D3
E80	$10^{-8}$	D5	E76	$10^{-8}$	S	C101	$10^{-6}$	D5
E84	$10^{-7}$	D4	E76	$10^{-7}$	S	E70	$10^{-8}$	S
E88	$10^{-7}$	S	E76	$10^{-7}$	D8	E77	$10^{-7}$	S
			E89	$10^{-6}$	D3	E88	$10^{-6}$	S
			E91	$10^{-5}$	D3	E92	$10^{-6}$	D3
						E106	$10^{-4}$	S

Each rat received the equivalent of 5 cc. growth and 1 mg. sodium glycocholate.

taneously with  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-7}$  and  $10^{-6}$  cc., respectively, only one ( $10^{-7}$  cc.) lived. Seventy-two hours after the single feeding  $10^{-6}$  cc. was fatal for each of two controls. Among the E rats, only one ( $10^{-8}$  cc.) died. The other rat injected with this dose, as well as two injected with  $10^{-7}$  cc., two with  $10^{-6}$  cc., three with  $10^{-5}$  cc. and two with  $10^{-4}$  cc. all survived.

Judging by these data it would seem as if the resistance to subcutaneous injection may also appear 48 hours after a single feeding. The remaining E rats of this group were injected intraperitoneally 96 hours after ingestion of the filtrate and survived 1, 10, 100 and 1000 fatal doses; one of two injected with 10,000 such doses also lived.

An effort was next made to measure simultaneously the immunizing value of feeding 0.1, 1, 5, and 50 cc. growth, in the form of Berkefeld filtrate of sodium glycocholate dissolved pneumococci, and of 50 cc. growth in the form of acid killed sedimented whole cells. The primary

TABLE XII

*Immunity to Subcutaneous and Intraperitoneal Injection Produced by Feeding a Single Dose of Sodium Glycocholate Dissolved Pneumococcus (Berkefeld Filtrate)*

Subcutaneous injection						Intraperitoneal injection		
48 hrs. after feeding			72 hrs. after feeding			96 hrs. after feeding		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
gm.	cc.		gm.	cc.		gm.	cc.	
C52	10 <sup>-8</sup>	D16	C59	10 <sup>-8</sup>	S	C67	10 <sup>-8</sup>	S
C56	10 <sup>-7</sup>	D12	C65	10 <sup>-8</sup>	S	C71	10 <sup>-8</sup>	S
C62	10 <sup>-7</sup>	S	C73	10 <sup>-7</sup>	S	C76	10 <sup>-7</sup>	D3
C64	10 <sup>-6</sup>	D3	C73	10 <sup>-7</sup>	S	C80	10 <sup>-7</sup>	D2
*E52	10 <sup>-8</sup>	D7	C75	10 <sup>-6</sup>	D15	C86	10 <sup>-6</sup>	D2
E55	10 <sup>-7</sup>	S	C76	10 <sup>-6</sup>	D3	C90	10 <sup>-6</sup>	D2
E60	10 <sup>-7</sup>	S	C78	10 <sup>-5</sup>	D2	C94	10 <sup>-5</sup>	D2
E68	10 <sup>-6</sup>	S	E54	10 <sup>-8</sup>	D16	E68	10 <sup>-7</sup>	S
			E63	10 <sup>-8</sup>	S	E74	10 <sup>-6</sup>	S
			E65	10 <sup>-7</sup>	S	E75	10 <sup>-6</sup>	S
			E66	10 <sup>-7</sup>	S	E75	10 <sup>-5</sup>	S
			E66	10 <sup>-6</sup>	S	E77	10 <sup>-5</sup>	S
			E68	10 <sup>-6</sup>	S	E82	10 <sup>-4</sup>	S
			E72	10 <sup>-5</sup>	S	E84	10 <sup>-4</sup>	S
			E73	10 <sup>-5</sup>	S	E84	10 <sup>-7</sup>	D2
			E73	10 <sup>-5</sup>	S	E89	10 <sup>-3</sup>	S
			E75	10 <sup>-4</sup>	S	E90	10 <sup>-2</sup>	D2
			E76	10 <sup>-4</sup>	S	E98	10 <sup>-2</sup>	D2

\* Heart's blood sterile. None of the others was examined.

Each rat received the equivalent of 5 cc. growth and 1 mg. of sodium glycocholate.

purpose was to learn whether feeding the equivalent of less than 5 cc. culture (bile salt dissolved) would prove as effective as feeding the equivalent of 5 cc. or more.

Feeding the acid killed bacteria from 50 cc. culture and the bile salt dissolved bacteria from the same quantity was included in order to learn whether there would

be any difference in effect produced by using what now appeared to be a much larger single dose of the latter than is necessary, and an equally large single dose of the former. The quantity fed (growth from 50 cc.) being definitely known to be more than sufficient, a poorer result with the acid killed bacteria could be interpreted as indicating an advantage in the use of a solution, due either to the ease with which such material can be absorbed or to the presence of the small amount of bile salt. By feeding all five groups at the same time, a more accurate comparison was expected than by dividing up the experiment. The organisms were grown in beef heart broth. Each of the several quantities required for the other groups of rats was centrifuged separately in order to make the dosage as accurate as possible. Since the quantity required for the animals receiving 0.1 cc. each was small, four times this amount was centrifuged and dissolved in four times the weight of sodium glycocholate needed, and after the material was filtered one-fourth of it was measured out, mixed with cracker meal and fed to each rat separately, just as for the remaining animals. The weight of bile salt ingested by each rat was 1 mg., except in the case of the animals fed the bile dissolved growth from 50 cc.; on account of the larger quantity of organisms, 4 mg. of sodium glycocholate were used. The animals were fasted for 18 hours before ingestion of the bacteria and for about 4 hours after. Twenty-four hours following the feeding of the filtrates the treated rats and controls were injected intraperitoneally with graded doses of pneumococci. The entire feeding part of the experiment was repeated twice more with new rats in order to measure the immunity after 48 and 72 hours. Table XIII gives the results. In the 24 hour test the amounts injected varied from  $10^{-8}$  to  $10^{-5}$  cc. in each of the six groups including the controls. There were no survivals either among the controls or those fed 0.1 cc. Among the animals in the 1 cc. group, two survived, one after an injection of  $10^{-8}$  cc., the other following  $10^{-7}$  cc. In the 5 cc. group, one survived  $10^{-6}$  cc., and in the 50 cc. group one rat which was injected with  $10^{-8}$  cc. lived. None survived among those fed the acid killed bacteria.

These results confirm the fact already observed that the bile salt dissolved bacteria from 50 cc. will produce neither an appreciably larger nor a more rapid response than the organisms from 5 cc.

The 48 hour test is difficult to interpret because of the apparent irregularity in the results.

The customary effect of feeding either 5 or 50 cc. is absent. The comparatively poor results may be due to the fact that just before the feeding all the animals were kept in a very small cage with only a small opening for air. The weather was very hot and humid and when the animals were taken out they were quite weak and prostrated and one had died. The 48 hour test with the 0.1 cc. and 1 cc. quantities, offers no aid in determining to what extent the equivalent of less than 5 cc. growth will prove of value in producing an immunity in this period of time. The

TABLE XIII

*Comparative Immunizing Values of a Single Feeding of Varying Quantities of Pneumococcus. (Berkefeld Filtrate)*

Test done 24 hrs. after feeding the filtrate			Test done 48 hrs. after feeding the filtrate			Test done 72 hrs. after feeding the filtrate		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
Controls								
gm.	cc.		gm.	cc.		gm.	cc.	
58	$10^{-8}$	D2	49	$10^{-8}$	D4	67	$10^{-8}$	S
66	$10^{-8}$	D3	57	$10^{-8}$	D3	74	$10^{-8}$	S
78	$10^{-7}$	D3	62	$10^{-7}$	D3	82	$10^{-8}$	D2
73	$10^{-7}$	D3	68	$10^{-7}$	D4	100	$10^{-8}$	D2
70	$10^{-6}$	D2	74	$10^{-6}$	D3	101	$10^{-7}$	D2
83	$10^{-6}$	D2	78	$10^{-5}$	D3	103	$10^{-7}$	D2
81	$10^{-5}$	D4				107	$10^{-6}$	D2
88	$10^{-5}$	D2				111	$10^{-6}$	D2
						112	$10^{-5}$	D2
Rats fed equivalent of 0.1 cc. culture								
48	$10^{-8}$	D3	45	$10^{-8}$	D3	75	$10^{-8}$	D3
63	$10^{-8}$	D2	46	$10^{-8}$	D3	74	$10^{-8}$	S
64	$10^{-7}$	D2	50	$10^{-7}$	D3	81	$10^{-7}$	D4
73	$10^{-7}$	D2	63	$10^{-7}$	D2	83	$10^{-7}$	D2
77	$10^{-6}$	D3	66	$10^{-6}$	D2	83	$10^{-6}$	D3
73	$10^{-5}$	D3	64	$10^{-6}$	D3	84	$10^{-6}$	S
90	$10^{-5}$	D2	69	$10^{-5}$	D2	91	$10^{-5}$	D2
84	$10^{-5}$	D2	72	$10^{-5}$	D3	93	$10^{-5}$	D2
			77	$10^{-4}$	D2	106	$10^{-4}$	D2
			72	$10^{-4}$	D3	103	$10^{-4}$	D4
			81	$10^{-3}$	D1	112	$10^{-3}$	D3
			92	$10^{-3}$	D2	120	$10^{-3}$	D2
			94	$10^{-2}$	D1			
			96	$10^{-2}$	D1			
Rats fed equivalent of 1 cc. culture								
60	$10^{-8}$	S	46	$10^{-7}$	D3	73	$10^{-8}$	S
66	$10^{-8}$	D3	50	$10^{-7}$	D3	75	$10^{-8}$	S
80	$10^{-7}$	D3	64	$10^{-6}$	D6	80	$10^{-7}$	S
78	$10^{-7}$	S	65	$10^{-6}$	D3	81	$10^{-7}$	S
77	$10^{-6}$	D2	66	$10^{-5}$	D2	*88	$10^{-6}$	D8
85	$10^{-6}$	D3	67	$10^{-5}$	D4	89	$10^{-6}$	D2
86	$10^{-5}$	D2	75	$10^{-4}$	D3	95	$10^{-5}$	S
84	$10^{-5}$	D2	75	$10^{-4}$	D2	101	$10^{-5}$	S
			77	$10^{-3}$	D3	102	$10^{-4}$	D2
			81	$10^{-3}$	D2	104	$10^{-4}$	D2
			90	$10^{-2}$	D2	120	$10^{-3}$	D3
			80	$10^{-2}$	D3	121	$10^{-3}$	D2

TABLE XIII—*Concluded*

Test done 24 hrs. after feeding the filtrate			Test done 48 hrs. after feeding the filtrate			Test done 72 hrs. after feeding the filtrate		
Wt.	Dose	Result	Wt.	Dose	Result	Wt.	Dose	Result
Rats fed equivalent of 5 cc. culture								
<i>gm.</i>	<i>cc.</i>		<i>gm.</i>	<i>cc.</i>		<i>gm.</i>	<i>cc.</i>	
59	10 <sup>-8</sup>	D3	45	10 <sup>-7</sup>	S	67	10 <sup>-8</sup>	S
67	10 <sup>-8</sup>	D3	58	10 <sup>-7</sup>	S	72	10 <sup>-8</sup>	S
70	10 <sup>-7</sup>	D4	68	10 <sup>-6</sup>	D4	82	10 <sup>-7</sup>	S
82	10 <sup>-7</sup>	D3	67	10 <sup>-6</sup>	D3	82	10 <sup>-7</sup>	S
85	10 <sup>-6</sup>	S	69	10 <sup>-5</sup>	S	89	10 <sup>-6</sup>	S
85	10 <sup>-6</sup>	D3	70	10 <sup>-5</sup>	D3	89	10 <sup>-6</sup>	S
86	10 <sup>-5</sup>	D3	75	10 <sup>-4</sup>	D2	92	10 <sup>-5</sup>	D2**
86	10 <sup>-5</sup>	D3	72	10 <sup>-4</sup>	D1	100	10 <sup>-5</sup>	S
			87	10 <sup>-3</sup>	D2	98	10 <sup>-4</sup>	D2
			86	10 <sup>-3</sup>	D1	104	10 <sup>-4</sup>	S
			87	10 <sup>-2</sup>	D2	110	10 <sup>-3</sup>	D4
			89	10 <sup>-2</sup>	D3	119	10 <sup>-3</sup>	D2
						126	10 <sup>-2</sup>	D1
Rats fed equivalent of 50 cc. culture								
58	10 <sup>-8</sup>	S	51	10 <sup>-7</sup>	S	*76	10 <sup>-8</sup>	D7
68	10 <sup>-8</sup>	D3	67	10 <sup>-6</sup>	S	83	10 <sup>-8</sup>	S
70	10 <sup>-7</sup>	D4	61	10 <sup>-6</sup>	S	87	10 <sup>-7</sup>	S
69	10 <sup>-7</sup>	D2	68	10 <sup>-5</sup>	S	94	10 <sup>-7</sup>	S
80	10 <sup>-6</sup>	D3	69	10 <sup>-5</sup>	D2	94	10 <sup>-6</sup>	S
67	10 <sup>-6</sup>	D5	70	10 <sup>-4</sup>	D2	101	10 <sup>-6</sup>	S
80	10 <sup>-5</sup>	D2	70	10 <sup>-4</sup>	S	101	10 <sup>-5</sup>	S
90	10 <sup>-5</sup>	D2	77	10 <sup>-3</sup>	D2	103	10 <sup>-5</sup>	S
			80	10 <sup>-3</sup>	D2	105	10 <sup>-4</sup>	D1
			83	10 <sup>-2</sup>	D1	110	10 <sup>-4</sup>	S
			86	10 <sup>-2</sup>	D1	110	10 <sup>-3</sup>	D7
						116	10 <sup>-3</sup>	D2
						119	10 <sup>-2</sup>	D1
Rats fed HCl acid killed pneumococcus equivalent to 50 cc. growth								
56	10 <sup>-8</sup>	D4	57	10 <sup>-7</sup>	D4	73	10 <sup>-8</sup>	S
68	10 <sup>-8</sup>	D2	63	10 <sup>-6</sup>	D3	74	10 <sup>-8</sup>	S
71	10 <sup>-7</sup>	D3	66	10 <sup>-5</sup>	S	82	10 <sup>-7</sup>	S
73	10 <sup>-7</sup>	D3	73	10 <sup>-5</sup>	D2	84	10 <sup>-7</sup>	S
77	10 <sup>-6</sup>	D3	73	10 <sup>-4</sup>	S	85	10 <sup>-6</sup>	S
73	10 <sup>-6</sup>	D3	77	10 <sup>-4</sup>	D2	89	10 <sup>-6</sup>	S
77	10 <sup>-5</sup>	D3	77	10 <sup>-3</sup>	D1	93	10 <sup>-5</sup>	S
88	10 <sup>-5</sup>	D3	78	10 <sup>-3</sup>	D3	106	10 <sup>-5</sup>	S
			81	10 <sup>-2</sup>	D2	110	10 <sup>-4</sup>	D2
			85	10 <sup>-2</sup>	D2	111	10 <sup>-4</sup>	S
						115	10 <sup>-3</sup>	D2
						118	10 <sup>-3</sup>	S
						137	10 <sup>-2</sup>	D2

\* *Pneumococcus* in heart's blood.

two survivors in the 1 cc. group in the 24 hour test, indicate that, had the 48 hour test not been irregular, a rather favorable degree of protection would have been

TABLE XIV

*Comparative Immunizing Value of a Single Feeding Equivalent to 1 Cc. and 5 Cc. of HCl Acid Killed Pneumococcus Growth*

48 hrs. after feeding			67 hrs. after feeding		
Wt.	Dose	Result	Wt.	Dose	Result
gm.	cc.		gm.	cc.	
*C86	$10^{-2}$	D3	*C62	$10^{-3}$	D2
C103	$10^{-8}$	D3	C81	$10^{-8}$	S
C105	$10^{-8}$	D5	C84	$10^{-7}$	D2
C111	$10^{-7}$	D2	C91	$10^{-6}$	D2
C106	$10^{-7}$	D2			
C118	$10^{-6}$	D2			

Rats fed equivalent of 1 cc.					
E66	$10^{-8}$	S	E51	$10^{-8}$	S
E52	$10^{-8}$	S	E69	$10^{-7}$	D2
E70	$10^{-7}$	S	E76	$10^{-7}$	S
E72	$10^{-7}$	S	E77	$10^{-6}$	D2
E72	$10^{-6}$	D3	E76	$10^{-6}$	S
E92	$10^{-6}$	D3	E88	$10^{-5}$	D2
E94	$10^{-5}$	D2	E85	$10^{-5}$	S
E95	$10^{-5}$	D2	E92	$10^{-4}$	D2

Rats fed equivalent of 5 cc.					
E38	$10^{-7}$	S	E51	$10^{-7}$	S
E45	$10^{-7}$	S	E54	$10^{-7}$	S
E54	$10^{-6}$	S	E57	$10^{-6}$	S
E54	$10^{-6}$	S	E57	$10^{-6}$	S
E55	$10^{-5}$	S	E57	$10^{-5}$	S
E60	$10^{-5}$	S	E57	$10^{-5}$	S
E75	$10^{-4}$	D4	E63	$10^{-4}$	D2
E70	$10^{-4}$	D2	E60	$10^{-4}$	S
E85	$10^{-3}$	S	E90	$10^{-3}$	D3

\* *Pneumococcus* in heart's blood.

obtained with this quantity at that time. The fairly extensive protection which the rats in the 1 cc. group possessed in the 72 hour test shows that this opinion is justified, and that the correct dose lies between 1 and 5 cc. The 5 and 50 cc.



amounts gave the usual 72 hour results. The acid killed bacteria produced the most uniform protection, but the difference in survivals between this group and the 5 cc. filtrate and 50 cc. filtrate groups is insufficient to warrant the conclusion that the results are very different.

There still remained the question whether acid killed pneumococci equivalent to 5 cc. and 1 cc. of growth would yield the same protection as equal quantities of bile dissolved organisms.

To obtain data on this point an experiment was performed in which acid killed bacteria equivalent to 1 cc. and 5 cc. growth were fed. Feeding took place for the two groups simultaneously, from the same culture, and following a 20 hour fast. Half the animals of each set were tested 48 hours and the other half, 67 hours after the ingestion of the organisms. The results, given in Table XIV, show that the growth from 1 cc. and from 5 cc. produce a resistance against 100 and 10,000 or more fatal doses, respectively after 48 hours. After 67 hours, the rats fed 1 cc. seem to have a greater resistance than these from the same group examined the day before, but it is irregular. The animals in the 5 cc. group respond about the same as those tested after 48 hours.

The results, as a whole, are about the same as were obtained when the Berkefeld filtrate of bile salt dissolved pneumococci was fed in equivalent quantities, and seem to show that no serious loss of active material takes place when the solution passes through the filter. It would also seem that the bile salt plays no part in the process of immunization.

#### DISCUSSION

It is apparent from the data accumulated here, that considerably smaller quantities of pneumococci than were used in the early experiments are sufficient to produce a high degree of protection. The organisms from 5 cc. culture or possibly less, either intact or dissolved in sodium glycocholate are enough. The increased resistance to the virulent pneumococcus is present, probably to the full extent, or almost so, 48 hours after the material is ingested and can occasionally be detected to a smaller degree at the end of 24 hours. It would seem that once a sufficient amount is fed, no great advantage accrues from the use of a larger dose so far as degree of immunity is concerned, although there may be an effect on its duration. The promptness and ease with which the increased resistance is created, makes the method

attractive when considered as a possible practical means of protecting human beings, especially where it is essential that the immunity be built up quickly. For the same reason it may be that feeding the pneumococcus in the very early stages of pneumonia will prove of value in favorably influencing the course of the disease. No apparent discomfort has been found to follow the ingestion, by human beings, of quantities of acid killed pneumococci which are much larger than would be required if dosage is calculated on the basis of weight. Measurements of the protective value of the sera of such individuals before and after the ingestion of the bacteria showed no increase in a few, while several others showed increases equivalent to 1, 10 and 100 fatal doses. Earlier experiments (4) have shown approximately similar results with the sera of rats immunized by feeding acid killed pneumococci. Judging from the results obtained with rats, it is uncertain whether the immunity need in all individuals be accompanied by an increase in the protective value of the serum. When an increase does take place, it is a relatively small one. The results so far obtained with humans are encouraging and this phase of the work is being continued. Additional work is also being done to determine the effect of the size of the dose, number of doses, and intervals, on the duration of the immunity. Recent experiments have shown that a single feeding, although producing a high degree of protection, does not create a very durable one, but that when the effect has worn off another single feeding will result in the reappearance of the high resistance.

In judging the relative values of the various forms in which the bacteria have so far been administered, the following facts stand out. The use of degraded avirulent cultures of Type 1 is of little value, only an occasional animal having been protected. The use of milk cultures is effective and may prove of value as a form in which the bacteria can be administered to humans. Sedimented bacteria, either whole or disrupted, or solutions of the organisms, permit of smaller actual bulk being used. Judging by the very favorable results obtained with bile dissolved cocci, it appears possible that even when intact cells are fed, they may be disrupted in the gastro-intestinal tract, and that the active portion undergoes solution before absorption takes place.

The fact that the dissolved contents of the pneumococcus are effec-

tive in eliciting an immunity when fed, permits one to regard an ingredient of the cell, rather than the intact organism as the active material. The variety of treatments to which either the whole or the dissolved cell has been submitted and then tested, allows one to draw up a tentative list of some of the properties of this substance. The results with the degraded pneumococcus, Type 1, indicate that the antigen is formed in relatively small quantities in this kind of cell. The antigen is water soluble and is uninjured by bile salts. Since the milk cultures were found effective, it would seem that heating at 60°C. for an hour in the presence of the lactic acid formed, did not destroy it. Earlier experiments (2) showed that heating the pneumococcus at 80°C. for 2 hours caused considerable destruction, although some of the rats fed the heated bacteria tolerated many fatal doses indicating that not all of the active ingredient was lost by this treatment. Contact with "N/12" hydrochloric acid for 3 hours at about 30°C. does not destroy it. Neither does desiccation. It is not destroyed by the gastrointestinal enzymes of the white rat. Attempts are now being made to determine additional properties of the substance.

It is still too early to say whether the active ingredient is one of the definitely known antigenic components of the pneumococcus cell (protein, carbohydrate).<sup>\*</sup> These two substances are supposed to exist united in an intact cell as a protein-carbohydrate complex. Solution of the cell is regarded as being accompanied by dissociation of the union and is known to destroy the property of eliciting agglutinins for the whole cell as well as precipitins for the carbohydrate fraction. The experiments described prove that solution does not interfere with the creation by feeding, of an increased resistance to the virulent organism. The few experiments which we have done showing that the protection is type specific (these need further support) might be interpreted as indicating either that a type specific substance other than the protein-carbohydrate complex is responsible or that this complex is not completely dissociated on solution and is responsible for the effects

<sup>\*</sup>Experiments done since writing the above show that feeding the specific polysaccharide of pneumococcus, Type 1, produces an increased resistance to the organism. In degree and time of appearance this resistance resembles that obtained by the methods described in this article. So far, however, the proportion of animals protected is smaller.

observed. The inability to find agglutinins and precipitins in the sera of immunized animals would argue against the latter conception.

#### SUMMARY

1. Feeding heat killed pneumococci grown in milk produces a fair degree of immunity.
2. Feeding acid killed degraded avirulent organisms produces little protection.
3. Feeding the desiccated, mechanically disrupted organisms creates a high degree of protection.
4. Feeding the Berkefeld filtrate of sodium glycocholate dissolved cells produce a high degree of immunity.
5. A single ingestion of this material equivalent to between 1 and 5 cc. growth is sufficient to protect a rat against 1000 to 10,000 fatal doses. Among rats fed the equivalent of 0.1 cc. an occasional one survives.
6. This degree of protection is present 48 hours after the feeding, and to a smaller extent exists in occasional animals at the end of 24 hours.
7. The treated animals are resistant to subcutaneous as well as intraperitoneal injections.
8. A single ingestion of hydrochloric acid killed pneumococci equivalent to between 1 and 5 cc. growth also protects within 48 hours against 1000 to 10,000 fatal doses intraperitoneally injected.
9. Reference is made to results obtained in preliminary experiments with human beings.

The author desires to record his appreciation to Dr. Wm. H. Park for his advice, and to Dr. Wm. G. Lyle and Dr. J. G. M. Bullova for their continued active interest during the course of the work.

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# THE EFFECT ON THE RATE OF UREA EXCRETION OF CARMINE DEPOSITION IN THE CELLS OF THE RENAL TUBULES\*

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A localized concentration of urea has been demonstrated in the epithelium of the proximal convoluted tubules of the kidney by Leschke (1) and this observation has since been confirmed by Oliver (2).

The method, which depends on the formation of an insoluble compound between urea and mercuric nitrate, is open to criticism on the basis of a possible lack of specificity. Chevallier and Chabanier (3) have found a similar high urea concentration in the cells of the convoluted tubules by means of a more delicate microchemical reaction, namely, the precipitation of dixanthyl urea by the interaction of urea and xanthidrol. Oliver (4) has repeated and extended the study of the distribution of urea in the kidney by means of the xanthidrol reaction. He found that extracellular crystals of dixanthyl urea were demonstrable in varying degree in all parts of the lumen of the renal vascular system. In the urinary lumen, sparse crystals were present in the glomerular capsule, more in the convoluted tubule, with still further accretions lower in the tubule. Crystals contained in cells were found only in the cortex, where the thick epithelium of the proximal convoluted tubule was filled with them. The number of crystals in the tubule cells gradually decreased so that there were very few in the terminal spiral portions of the proximal tubule and in the other divisions of the renal tubule intracellular crystals were nearly always absent. These observations have since been confirmed by a number of investigators (5, 6, 7) who have used the same method.

The question arises as to whether the source of this high urea concentration in the cells of the proximal convoluted tubules is absorption from the lumen of the tubule or excretion from the blood. In the light of other knowledge relating to the mechanism of urine formation

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Oliver (4) concluded that the second source was the more likely, since the assumption of an excretion by the tubule cells was the most direct conclusion from the evidence then at hand. More recently, however, the importance of absorptive activities has been emphasized by Oliver and Shevky (8) in experiments in simpler kidneys and with more direct methods, so that it would seem appropriate to reexamine the question. If it were possible to occlude the tubule cells or even decrease their function without affecting the other elements of the kidney the effects should indicate whether the tubules are removing urea from the urine by absorption or excreting it into the tubule lumen from the blood stream. The experiments which follow were therefore devised.

After the administration of a dye such as carmine by the vascular route, a fine granular deposit of the dye takes place in the tubule cells and, as has been shown by Suzuki (9), this deposit is most marked in the cells of the proximal convoluted tubules. It seemed possible that an excessive storage of the dye in these cells might interfere with their normal function. Just how much "blocking" there is may be questioned, but the histological appearance of these cells indicates that they have been damaged by the deposition of the dye and this alone might be expected to alter their normal activity. On the other hand, no histological evidence of damage to the other structures of the kidney can be found. Consequently the rate of urea excretion has been determined in a group of rabbits before and following intense staining of the renal tubule cells by intravenous injections of lithium carminate. Certain special conditions have been found (10) under which the rate of urea excretion in any one subject varies only with the blood urea concentration so that the excretory ratio: 
$$\frac{\text{Rate of urea excretion}}{\text{Blood urea concentration}}$$
 becomes a constant. In the experiments to be described here any increase or decrease in the rate of urea excretion as a result of the experimental variable may then be measured by the changes in this "ratio."

### *Methods*

Healthy male rabbits were used. After abstinence from food for 18 hours, 1 gm. of urea per kilo body weight was given in 4 per cent solution by stomach tube

and every hour thereafter for 5 hours the tube was again introduced and 15 ml. of water per kilo body weight were administered. Three hours after giving the urea the bladder was catheterized and washed. At approximately hourly intervals thereafter three collections of urine were made by catheterization and after each collection the bladder was washed out. Samples of blood were obtained by puncture of the left heart at the middle of these three periods of urine collection. The urea in the urine and the blood was determined by Addis' methods (11) and the average ratio:  $\frac{\text{Urea in 1 hour's urine}}{\text{Urea in 100 ml. blood}}$  was calculated.

Beginning on the day that the first "ratio" was determined and every other day thereafter each rabbit was given 4 ml. per kilo of a 2.5 per cent\* solution of carmine in half saturated lithium carbonate until a total of 8 doses containing a total of 0.8 gm. carmine per kilo had been given. Three days after the last carmine injection and 18 days after the first one, the rate of urea excretion was again determined by the same procedure as has already been described. The following is a typical protocol:

Rabbit 1. Weight, 2800 gm.

November 21, 1924.

12:30 p.m.—70 ml. of 4 per cent urea solution in water by stomach tube.

1:33 " —42 ml. of water by stomach tube.

2:31 " —" " " " " " "

3:33 " —" " " " " " "

4:36 " —" " " " " " "

5:32 " —" " " " " " "

The times given in the following table are those at which the washing of the bladder was completed and the times at which blood was collected.

Time	Urine urea	Time	Blood urea	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$
	<i>mg. per kilo per hr.</i>		<i>mg. per 100 ml.</i>	
3:30-4:32	257	4:00	120	1.30
4:32-5:30	148	5:02	105	1.41
5:30-6:30	148	6:00	94	1.57
Average (before carmine).....				1.42

\* The carmine solutions showed peculiar differences. The first dye was a pre-war product made in Germany. Excellent results were obtained with this but they could not be duplicated with domestic products of recent manufacture. The latter proved to be toxic and killed numerous animals but after the solution had remained in the laboratory in a loosely stoppered bottle for over 2 years it also was without obvious ill effects. When freshly prepared, the solution has a strong acrid odor resembling oil of cloves and this disappeared almost entirely on standing.



11.2 ml. of 2.5 per cent carmine in half saturated lithium carbonate solution were injected intravenously through the marginal ear vein on November 21, 23, 25, 27, 29, and December 1, 3, and 5.

December 8, 1924.

Urea solution and water were administered as on November 21.

Time	Urine urea	Time	Blood urea	Ratio: $\frac{\text{Urine urea}}{\text{Blood urea}}$
	<i>mg. per kilo per hr.</i>		<i>mg. per 100 ml.</i>	
3:35-4:36	189	4:05	130	1.38
4:36-5:34	192	5:06	116	1.64
5:34-6:35	196	6:06	112	1.76
Average (after carmine).....				1.59

### RESULTS

The results of the 20 experiments are given in Table 1. In every animal except one the rate of urea excretion was greater after the renal tubules had been stained with carmine than before. In the single exception there was no appreciable change. There is considerable variation in the degree of increase in the rate of urea excretion for the different rabbits in the group but this might be expected as a result of the variable staining of the renal tubules, the kidneys of some rabbits containing appreciably more carmine than those of others.

The actual average increase in the ratio:  $\frac{\text{Rate of urea excretion}}{\text{Blood urea concentration}}$  was 0.17, a 13.8 per cent increase in the rate of urea excretion. Although not of large magnitude the increase considered from a statistical point of view is significant. The expected difference between the average ratios before the carminization and those obtained afterward is only 0.04, while the observed difference is 0.17. The chances then are that the increase is due to the known experimental variable. There may be some question concerning the applicability of such statistical method to data of the nature of those with which we are concerned here. However in two other groups of rabbits of 10 and 12 animals each, in which urea excretory ratios were determined as in the carmine experiments and repeated within about the same period of time but without the introduction of an experimental variable, the actual average change was plus 2 per cent for one group and minus 3 per cent for the other.

In practically every experiment the urine volumes during the three ratio periods were appreciably greater after the carmine injections than those obtained after the administration of the same amount of urea

TABLE 1

No.	Weight	Urine volume cc. per kilo per hour		Urea ratio: Urine urea rate Blood urea conc.		Ratio increase	
		Before carmine	After carmine	Before carmine	After carmine	Actual	Per cent
	gms.						
1	2800	12.3	15.5	1.42	1.59	0.17	10.6
2	2700	16.4	23.9	1.36	1.50	0.14	9.3
3	2100	21.2	23.9	1.34	1.45	0.11	7.6
4	2200	20.9	35.2	1.35	1.80	0.45	33.3
5	2250	15.6	20.8	1.00	1.37	0.37	37.0
6	2500	14.8	14.5	1.18	1.38	0.20	16.9
7	2500	21.4	14.6	1.39	1.32	0.00	0.0
8	2400	16.6	20.0	1.24	1.39	0.15	12.1
9	2600	14.6	17.2	1.40	1.47	0.07	5.0
10	2600	14.6	15.2	1.00	1.10	0.10	10.0
11	3100	12.1	13.0	1.30	1.31	0.01	0.1
12	2200	11.4	14.7	1.39	1.46	0.07	5.0
13	2000	11.1	19.5	1.10	1.37	0.27	24.5
14	2000	12.2	13.7	1.02	1.38	0.36	35.2
15	1600	7.9	18.7	1.48	1.95	0.47	31.8
16	1600	6.0	12.9	0.89	1.03	0.14	15.7
17	1900	11.7	16.0	0.97	1.00	0.03	3.0
18	1900	12.3	17.0	1.25	1.49	0.24	19.1
19	2500	13.3	16.6	1.19	1.26	0.07	5.9
20	2500	9.7	15.3	1.23	1.30	0.07	5.7
Mean.....	2300	13.8	17.9	1.23	1.40	0.17	14.4
Standard deviation....		4.02	4.94	0.17	0.21		
Probable error.....		0.61	0.75	0.03	0.03		
Coefficient of varia- bility.....		29.1	27.6	13.9	14.9		
Actual difference of the means.....		4.10		0.17			
Probable difference of the means.....		0.96		0.04			

and water in the three 1-hour periods carried out before. Although it may have some significance this increased water excretion may be only secondary to the increased amount of urea excreted after the carmine injections.

## DISCUSSION

The results do not support the idea that the high urea concentration in the cells of the proximal convoluted tubules represents urea passing from the blood into the tubules in the secretion of urea by these cells. On the contrary they lend weight to the theory that all of the urea comes from the glomerular filtrate and that part of it is absorbed by the tubules.

It has been demonstrated (12) in the perfused frog's kidney that urea is excreted by glomerular filtration and the evidence is strongly suggestive of tubular reabsorption. More indirect experiments showed that in all probability urea is likewise excreted through glomerular filtration by the mammalian kidney. This supports the view that the high urea concentration in the tubule cells represents urea reabsorption. Other experiments to be reported elsewhere, in which the excretory ratios of creatinine and urea were compared, also lend support to this contention.

Under certain "standard conditions," conditions which it is reasonable to suppose would be most apt to cause all of the renal elements to become active and result in a state of full renal activity, a remarkable constancy in the ratio: 
$$\frac{\text{Urine urea rate}}{\text{Blood urea concentration}}$$
 has been demonstrated (13). This relation between the rate of urea excretion and the urea concentration in the blood is exactly what would be predicted if urea is eliminated entirely by glomerular filtration, provided that it is not reabsorbed and the rate of filtration is constant. In fact, if no reabsorption of urea takes place under the "standard conditions," the ratio  $\times 100$  would represent the amount of glomerular filtrate in cubic centimeters per hour. Although we are presenting evidence which points to a reabsorption of urea, certain observations (14) have been interpreted to indicate that urea might be reabsorbed ordinarily but that under the "standard conditions" this urea reabsorption disappears. It is, however, under the "standard conditions" that urea reabsorption appears to have been demonstrated. It then becomes necessary to reconcile these observations. To do this one must assume that if urea is reabsorbed, the reabsorption rate under "standard conditions" is directly proportional to the blood urea concentration,

or consequently the observed urine urea rate, an hypothesis which after all is not unreasonable.

#### SUMMARY

The evidence that urea is present in high concentration in the cells of the convoluted tubules of the kidney and is passing through these cells has been reviewed. Following the deposition of a large amount of carmine in the cells of the convoluted tubules the rate of urea excretion, as measured by the relation of the rate of urea excretion to the blood urea concentration, is increased. This is interpreted as due to blockage or damage to the cells of the convoluted tubules which interferes with their usual function of absorption of urea and other substances from the glomerular filtrate and hence increases the amount of urea which reaches the bladder urine at a given blood urea concentration.

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# STUDIES ON THE COMMON COLD

## III. THE UPPER RESPIRATORY FLORA OF INFANTS

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In recent years investigators of the common cold have realized the importance of ascertaining the normal basal flora of the human nasopharynx before drawing any conclusions as to the rôle played by any given microorganism in disease. From the observations of Williams (1), Bloomfield (2) and Jordan (3), it became apparent that many of the bacteria formerly thought to be responsible for the production of colds might occur as seemingly harmless inhabitants of the throats of healthy adults. Shibley, Hanger and Dochez (4), studying a group of thirteen normal adults throughout an entire winter and comparing their basal flora with that occurring during their colds, arrived at the conclusion that the potential pathogens may be present in healthy throats, that no bacteria can be found in early cold cultures to which an etiological rôle can be assigned, but that certain organisms probably play a part as late or secondary invaders. Exception may possibly be noted, however, in the case of certain geographically unusual epidemics as described by Burky and Smillie (5); in the metropolitan centers, on the other hand, these conclusions, supported later by Noble, Fisher and Brainard (6), hold good. By inference, then, the cause of the acute infectious cold, remaining undetected by ordinary bacteriological methods, probably falls into the category of an unknown virus to be sought by the methods of Foster (7), Olitsky and Gates (8), and later by Dochez, Shibley, and Mills (9).

The present study of the nose and throat flora of infants was undertaken with two ends in view: in the first place, no observations on the bacteriology of the pharynx of the newborn could be discovered in the literature, and secondly it was felt that the importance of the various bacteria could be more clearly evaluated if studied in the simplest possible host. The infant, unaltered by long contact with a hostile environment, seemed an ideally simple medium for the study of upper respiratory disease.

*Methods.*—Cultures of the nose were made by passing a sterile cotton swab on fine copper wire through the naris as far as possible; those of the throat were obtained by swabbing the visible portion of the oropharynx. Swabs were immediately rubbed on freshly-poured 5 per cent rabbit's-blood-agar plates and streaked with a platinum wire. The plates were incubated aerobically and read at the end of 20 to 24 hours. Identification of the organisms was made by colony appearance, staining and morphology, with more complete bacteriological study where indicated. Diphtheroid bacilli were taken as a group, as were the Gram-negative cocci. Streptococci were classified as green, hemolytic and indifferent (alpha, beta, and gamma types). Members of the colon group were tested for sugar fermentation. All pneumococci were typed and injected into a mouse to obtain a rough measure of virulence, those strains of which 0.1 cc. of an 18 hour blood broth culture killed being considered virulent. Staphylococci were listed according to pigment formation and a large staphylococcus-like organism growing in gray colonies was designated "large Gram-positive coccus." Pfeiffer's bacillus was carefully searched for and, if hemolytic, distinguished as "Bacillus X."

TABLE I

*First Positive Throat Culture—Predominating Organism*

30 newborn babies

Green streptococcus	Indifferent streptococcus	<i>Staphylococcus albus</i>	<i>Staphylococcus aureus</i>	Colon group	Diphtheroid group	Large Gram-positive coccus
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
37	23	2.7	3	3	3	3

*Results.*—Observations were first made on 30 newly-delivered babies in Sloane Hospital for Women. In the beginning of the series, cultures of the oral contents were actually obtained in the delivery room, but when these were found to be almost invariably sterile the practice was discontinued and the first throat culture was made a few hours after birth. In 80 per cent of cases these initial throat cultures showed no growth and until the first feeding the pharynx ordinarily remained sterile. After the first feeding, however, the initial flora had regularly made its appearance and the pharynx never regained its original sterility. The first predominating organism to appear was ordinarily the non-hemolytic streptococcus (60 per cent), with staphylococci next in frequency (30 per cent). Table I shows the relative frequency with which these organisms predominated in the first positive cultures to be obtained.

Successive cultures were then made during the first 2 weeks of life, that is to say, during the infant's stay in the maternity hospital. Profuse growth was almost always obtained from the throat. Over the whole series of these cultures indifferent streptococci were more prominent than the green producing type. *Staphylococcus albus* was almost always present, less frequently predominant than indifferent streptococci, but more frequently than green. In order of diminishing importance the following organisms were also observed: large Gram-positive cocci, *Staphylococcus aureus*, colon bacilli, diphtheroid bacilli, and Gram-negative cocci (see Tables III and IV). Pfeiffer's bacillus, pneumococcus and hemolytic streptococcus were never observed in the entire group in the first 2 weeks of life—in spite of the fact that during the whole course of the study the incidence of Pfeiffer's bacillus in the

TABLE II

*First Positive Nasal Culture—Predominating Organism*

25 cases

<i>Staphylococcus albus</i>	<i>Staphylococcus aureus</i>	Diphtheroid group	Large Gram-positive coccus	Indifferent streptococcus	Green streptococcus
per cent	per cent	per cent	per cent	per cent	per cent
76	8	4	4	4	4

normal hospital population ran well over 50 per cent. Thus the basal flora of the normal infant's pharynx in its first 2 weeks of life presents one striking difference from that of the normal adult's: the potential pathogens are never found. An additional note is the relatively much lower incidence of the Gram-negative cocci.

Nasal cultures were also made and showed no growth on the first day of life in 85 per cent of cases. In 40 per cent they remained sterile on the second day as well. Following this, organisms were invariably present, although growth from the nose, when established, was rarely abundant. The organism to predominate in the first positive culture was the *Staphylococcus albus* in most instances.

During the first 2 weeks of life the staphylococci were by far the most prominent organisms in the nose. Diphtheroid bacilli ranked next in incidence with large Gram-positive cocci following. Strepto-



cocci were much less common than in the throat. Colon bacilli were quite infrequent (see Tables V and VI). As in the throat cultures, the potential pathogens were never found and the same differences existed between the nasal cultures of the newborn and the normal adult as were observed in the throat. That is to say, pneumococcus, Pfeiffer's bacillus and hemolytic streptococcus were never present in the nose during the first 2 weeks of life.

TABLE III

*Percentage Incidence of Different Organisms in Throat Cultures*

	Indiff. strep.	<i>Staph. albus</i>	Green strep.	Large Gram-pos. cocci	<i>Staph. aureus</i>	Colon group	Diphtheroid bac.	Gram-neg. cocci	<i>B. Pfeifferi</i>	Pneumococcus	Hem. strep.	Bacillus "X"
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
A. 30 normal infants during first 2 weeks of life No. of cultures, 155	78	92	44	25	23	16	7	4	0	0	0	0
B. 28 normal infants between 4 and 8 months of age	61	46	100	36	18	7	7	71	53	0	11	7
C. 21 infants early in the course of their first cold Average age, 3 months	57	62	91	38	24	10	24	38	14	0	0	0
D. 14 infants with recurrent colds Average age, 7 months	21	14	100	29	7	0	21	71	57	14	0	7

Following discharge from the maternity wards, as many of these infants as possible were followed from time to time, particularly with a view to obtaining cultures during their first colds. In addition, other dispensary babies were cultured and the combined findings of the entire series are given in Tables III, IV, V, and VI, showing percentage incidence and percentage predominance of the various organisms.

The infants were divided into four groups. Group A—normal infants in the first 2 weeks of life—has already been discussed.

Group B is next introduced for comparison to show what changes take place as the infant grows older. It consists of 28 babies from 4 to 8 months of age, all entirely free from colds. Certain important differences are at once apparent in the throat cultures. For the first

TABLE IV

*Percentage Predominance of Different Organisms in Throat Culture*

	Indiff. strep.	<i>Staph. albus</i>	Green strep.	Large Gram-pos. cocci	<i>Staph. aureus</i>	Colon group	Diphtheroid bac.	Gram-neg. cocci	<i>B. Pfeifferi</i>	Pneumococcus	Hem. strep.	Bacillus "X"
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
A. 30 normal infants during the first 2 weeks of life	41	32	19	5	1	5	1	0	0	0	0	0
No. of cultures, 155												
B. 28 normal infants between 4 and 8 months of age	7	0	78	0	0	0	0	14	3	0	0	0
C. 21 infants early in the course of their first cold	14	0	76	0	0	0	0	0	10	0	0	0
Average age, 3 months												
D. 14 infants with recurrent colds	14	0	86	0	0	0	0	0	0	0	0	0
Average age, 7 months												

time the potential pathogens were found. It will be observed that Pfeiffer's bacillus was present in the majority of these normal infants' throats (53 per cent), and that hemolytic streptococci were noted in 11 per cent. The throat cultures also showed an increase in prominence of the green-producing streptococcus and the Gram-negative cocci, at the expense of indifferent streptococci.

In the nose two potential pathogens also made their first appearance

and while *B. Pfeifferi* was rare, pneumococci appeared in 14 per cent of the cultures and in 11 per cent were actually predominant. (Nine of the infants never had colds; the remaining 19 gave a history of one or more. No significant differences in the flora were noted except a slightly higher incidence of Gram-cocci in the throats of the latter. The significance of the changes observed in this group is interesting; it appears that in the first 6 months of life the newborn's flora steadily approaches that of the normal adult and that infants can actually harbor

TABLE V

*Percentage Incidence of Different Organisms in Nasal Cultures*

	<i>Staph. albus</i>	Diphtheroid bac.	<i>Staph. aureus</i>	Large Gram- pos. cocci	Indif. strep.	Green strep.	Colon group	Gram-neg. cocci	<i>B. Pfeifferi</i>	Pneumococcus	Hem. strep.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
A. 30 normal infants during first 2 weeks of life No. of cultures, 103	93	43	32	21	15	4	3	1	0	0	0
B. 28 normal infants between 4 and 8 months of age	79	46	22	68	14	7	7	18	7	14	0
C. 21 infants early in the course of their first cold Average age, 3 months	62	81	48	43	14	10	5	14	14	0	0
D. 14 infants with recurrent colds Average age, 7 months	57	29	21	57	7	7	14	14	21	43	0

the pathogens with impunity. In other words, the mere acquisition of a potentially pathogenic organism by an infant does not necessarily produce any symptoms whatsoever. Pneumococcus, Pfeiffer's bacillus, and hemolytic streptococcus can apparently live as harmless inhabitants of the upper respiratory tract of an entirely normal infant in the first 6 months of life.

Group C represents 21 infants studied early in the course of their first cold. From a clinical standpoint these first colds are usually very mild.

The child sneezes and coughs; there may or may not be a small amount of thin nasal discharge and constitutional symptoms, as a rule, are absent. There is no real difference between the flora found in these first mild colds and that of the normal group. The potential pathogens, while present, were actually less conspicuous than in the normals, a fact possibly attributable to the younger average age of these infants. In other respects the findings are strikingly similar; to be sure, the incidence of diphtheroid bacilli in the nose was high in the cold group, but it predominated less frequently. From this comparison it

TABLE VI

*Percentage Predominance of Different Organisms in Nasal Cultures*

	<i>Staph. albus</i>	Diphtheroid bac.	<i>Staph. aureus</i>	Large Gram- pos. cocci	Indiff. strep.	Green strep.	Colon group	Gram-neg. cocci	<i>B. pertussis</i>	<i>Pneumococcus</i>	Hen. strep.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
A. 30 normal infants during first 2 weeks of life No. of cultures, 103	67	5	18	5	4	0	2	0	0	0	0
B. 28 normal infants between 4 and 8 months of age	25	22	7	22	4	0	4	7	0	11	0
C. 21 infants early in the course of their first cold	19	10	24	24	10	0	5	0	5	0	0
D. 14 infants with recurrent colds	21	7	7	7	0	0	0	7	14	36	0

becomes apparent that one cannot usually assign a causative rôle to any organism in the first colds of infancy, and it might be justifiable to conclude that the process is similar to the acute infectious cold in the adult, mention of which has already been made.

Group D, composed of 14 infants, on the average slightly older (7 months), includes only those who gave a history of recurrent colds, cultures being taken during an exacerbation or reinfection. Clinically speaking, many of these infants were quite separate from the "first cold" group; their symptoms were often severe, with purulent nasal discharge, loss of appetite and fever. Their throat flora, however,

was similar to that of the normal group excepting for the first appearance of pneumococci (14 per cent). On the other hand, a striking change was noted in the nose. Pneumococci were found in 43 per cent, actually predominating in 36 per cent. Pfeiffer's bacillus was noted in 21 per cent and it predominated in 14 per cent. Most of the infants showing a heavy growth of either of these organisms from the nose presented a picture similar to that of chronic winter colds in adults, with purulent nasal discharge and the severer symptoms mentioned above. It is not improbable that this represented a secondary invasion of the respiratory tract by these pathogenic organisms, with chronic lodgment, perhaps in the maxillary antrum, and frequent exacerbations precipitated by various causes. (The pneumococci fell into Group IV and most of them were "virulent.")

#### CONCLUSIONS

1. The upper respiratory tract is sterile at birth.
2. In the first 2 weeks of life the infant acquires a basal flora comparable to that of adults except that the potential pathogens are absent.
3. During the ensuing months the potential pathogens may appear without giving rise to symptoms and by 8 months the infant's flora is entirely comparable to the adult's.
4. There is no evidence of a specific bacterial incitant for the first colds of infancy.
5. In infants with recurrent colds, secondary infection of the nose with pneumococci or *B. Pfeifferi* probably plays a part.

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# REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

## IV. THE DEVELOPMENT OF SKIN REACTIVITY TO DERIVATIVES OF PNEUMOCOCCUS

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Many reports have been made concerning the occurrence of reactions in the skin following the intracutaneous injection of Pneumococcus products in pneumonia patients at the time of crisis, or after the crisis, or into guinea pigs after they had previously received injections of the organism or its derivatives. These reports indicate that the reactivity of the skin to certain derivatives of Pneumococcus may become increased after the patient or animal has undergone infection, or after they have been injected with certain products of Pneumococcus parenterally. It has usually been assumed, therefore, that a state of increased reactivity of the skin to Pneumococcus protein may occur, probably analogous to the state of hypersensitiveness which arises following the administration of such a foreign protein as egg albumin. The exact conditions under which this state of altered reactivity of the skin may occur are not as yet well understood. Moreover, the relation of this condition of increased skin reactivity to the general state of altered reactivity or "allergy" is still as obscure in the case of pneumococci and their products as it is in the case of most other reacting agents. An understanding of the underlying principles can probably be arrived at only after the various phenomena have been carefully observed and recorded under a great variety of conditions and in various animals. Experience has shown that the various phenomena thought to be related to the general condition of hypersensitiveness, such as reactions occurring in the skin following the injection of foreign substances, the ophthalmic reaction, anaphylaxis in the guinea pig, increased reaction

of the musculature of the uterus, serum sickness, etc., usually cannot all be elicited in the same animal. It is also probable that they are not necessarily all manifestations of the same biological process.

In the present study an investigation has been made of the skin reactions in rabbits following the injection of certain derivatives of the pneumococcal cells.

This work is a part of the investigation of the changes which take place in rabbits following the repeated intracutaneous injections of suspensions of heat-killed pneumococci, some of the results of which have been reported in previous communications (1, 2, 3). Consequently most of the skin tests have been made in rabbits that have been previously treated in this way, but for the sake of comparison, skin tests have also been performed in normal rabbits and in rabbits treated in various other ways. The derivatives of pneumococci employed in the tests have been the so-called "nucleoprotein," the supernatant fluid after precipitation of the "nucleoprotein" from the bacterial extract and to a less extent the carbohydrate fraction, the specific polysaccharide. The method of preparing the first two derivatives was given in a previous communication (1). The specific polysaccharides were obtained from Dr. Avery and the method of their preparation has been fully described (3). The rabbits were prepared by depilation of the skin over the abdomen as previously reported (1).

The first skin tests were made in rabbits which had received repeated intracutaneous injections of suspensions of heat-killed pneumococci. About 3 weeks after the animal had received the last of 8 to 12 intracutaneous injections, tests were performed by injecting into the skin a solution of "nucleoprotein." The volume of fluid injected for each test was 0.2 cc. and the amount of the test substance was measured by the determination of the nitrogen present, considering this as all derived from protein. Control tests have been made repeatedly in normal animals that had received no preliminary treatment, and in none of these animals have reactions in the skin been observed following the injection of amounts of "nucleoprotein" two to four times larger than any doses employed in testing the previously treated animals.

It has been found that when 0.2 cc. of a solution of "nucleoprotein" is injected into the skin of a rabbit which has previously received intracutaneous injections of heat-killed pneumococci, within a few hours the skin about the site of injection becomes red, raised and edematous. The reactions reach a maximum in 24 to 48 hours, then begin to fade

and usually disappear within 3 to 4 days. The degree of reaction depends to a considerable extent on the amount of the protein contained in the solution. Reactions may occur with amounts of protein as small as 0.0025 mg. Usually larger amounts 0.2 to 0.6 mg. have been injected. Under the latter circumstances the area of skin involved in the reaction measures on an average 1.5 to 2 cm. in diameter. A certain area of erythema not infrequently is seen but a breaking down of the skin has never been observed.

The reactivity of the skin to the supernatant fluid after precipitation of the "nucleoprotein" from the bacterial extract was also studied in rabbits following repeated intracutaneous injections of heat-killed pneumococci. A solution of the concentrated bacteria was made by repeated freezing and thawing. The "nucleoprotein" was then precipitated out with acetic acid and the resulting supernatant fluid, adjusted to slightly acid to litmus, was boiled over a free flame for 10 minutes. This extract gave none of the usual qualitative tests for protein. In the doses employed in this study, it was found to possess a primary toxicity, so that in over half the normal rabbits, it caused a local reaction in the skin consisting of an erythematous blush with thickening. In these animals the reaction disappeared in 1 to 3 days. In rabbits which had previously received repeated intracutaneous injections, the skin reaction following the administration of this extract was more marked. The area of the skin involved was larger, the elevation was more marked, and a central area of ecchymosis, which was never seen in the untreated animals, was not infrequently observed. In some instances the skin over the lesion broke down. Moreover, in these animals the reactions lasted longer, disappearing in 3 to 5 days.

In some of the rabbits the reactivity of the skin to the specific soluble substances, the polysaccharides, was also studied. Dilutions of 1-1000, 1-10,000 and 1-25,000 of the carbohydrates were injected in the skin about 3 weeks after the last intracutaneous injection of heat-killed pneumococci. No reactions were seen following the intracutaneous introduction of the carbohydrate derived from homologous or heterologous types of *Pneumococcus*.

In terms of bacterial specificity, then, the skin reactions observed in rabbits after repeated intracutaneous injections of heat-killed



pneumococci are not type-specific, but species-specific. The skin reactions to the derivatives of *Pneumococcus* are elicited by materials which are not associated with type-specificity, and in all instances, the test reagents were derived from a type other than that employed during the repeated injections.

*The Occurrence of the Skin Reaction Following Repeated Injections, by Different Routes, of Pneumococci or Their Products*

Experiments were next undertaken to determine whether rabbits which have received repeated intravenous or intraperitoneal injections of dead pneumococci exhibit an increased reactivity of the skin, just as do animals which have received repeated intracutaneous injections.

TABLE I

*The Incidence of the Skin Reaction to Pneumococcus Protein in Rabbits Following Repeated Injections of Pneumococcus or Its Derivatives*

Material injected	Route of injection	Number of rabbits	Skin test	
			Positive	Negative
Heat-killed <i>Pneumococcus</i> .....	Intracutaneous	60	60	—
Heat-killed <i>Pneumococcus</i> .....	Intravenous	40	40	—
Heat-killed <i>Pneumococcus</i> .....	Intraperitoneal	2	2	—
Nucleoprotein.....	Intracutaneous	8	8	—
Nucleoprotein.....	Intravenous	8	8	—
Supernatant fluid from bacterial extract.....	Intracutaneous	4	4	—

It was found that in the former animals, skin reactions of the same nature might follow the injections of the derivatives of pneumococci. The data are presented in Table I. It is seen that 40 rabbits received intravenous administration, and two, intraperitoneal injections of heat-killed bacteria. All the rabbits were found to be skin reactive.

While skin reactions to the nucleoprotein were obtained with regularity in this group of rabbits following intravenous injections of pneumococci, it has been found that when skin tests are undertaken, as here, 3 weeks after the last injection of organisms, the skin reaction may not be of great severity or in some cases it may be absent.

In another experiment, the skin reactivity was studied in rabbits which had previously received repeated injections of the "nucleoprotein," also in rabbit which had received repeated injections of the supernatant fluid after precipitation of the "nucleoprotein" from a bacterial extract. The data of this experiment are given in Table I. It is seen that 8 animals received the "nucleoprotein" intracutaneously and 8, intravenously, while 4 rabbits received the supernatant fluid from the bacterial extract intracutaneously. The animals were tested for skin reactivity 19 days after the last injection of these materials. Skin reactions, as described above were obtained in all the animals.

It may be concluded, therefore, that regardless of the material employed for the injections and irrespective of the route of administration of the material, skin reactivity resulted in all the rabbits.

#### *Relation of the Skin Reaction to Resistance to Infection*

In a previous publication (3), it was shown that following repeated intracutaneous injections of heat-killed pneumococci, rabbits acquire a marked degree of resistance to infection by *Pneumococcus* of homologous or heterologous types. It was also shown that following similar injections with the "nucleoprotein" and supernatant fraction of the bacterial extract of *Pneumococcus*, rabbits do not become resistant to infection. In the present study it has already been pointed out that skin reactivity to derivatives of *Pneumococcus* is found to occur in rabbits which have been injected by any route with the intact cell or its protein derivatives. It is obvious, therefore, that skin sensitivity occurs with equal frequency in animals which are resistant to infection (*i.e.*, following injections with the intact cell) and in non-resistant animals, as for example, following repeated injections with derivatives of *Pneumococcus*.

#### *Relation of the Skin Reaction to Circulating Antibodies*

It has already been stated above that the skin reaction described in this communication cannot be related to type-specificity. The skin reactions are elicited by a species-specific reagent in the absence of demonstrable type-specific antibodies, as is seen, for example, in the rabbits which receive repeated intracutaneous injections of heat-killed pneumococci (1). On the other hand, the skin reaction to the "nucleo-

protein" always occurs in the presence of circulating species-specific antibodies, and in a general way the intensity of the reaction to a given quantity of protein varies with the titre of species-specific antibodies in the sera of the animals.

#### DISCUSSION

That increased reactivity of the skin to *Pneumococcus* or its products may be induced artificially in animals or may accompany natural infection by *Pneumococcus* has been pointed out by a number of authors.

Mackenzie and Woo (1) described the development of a skin reaction to bacterial protein following intracutaneous injections of an alkaline extract of *Pneumococcus*. Zinsser and Tamiya (12) reported that guinea pigs sensitized by injections of pneumococci or the bacteria protein gave skin reactions to autolysates and they concluded that this reaction was not related to type-specificity. Later Zinsser and Grinnell (13) induced skin sensitization in guinea pigs to autolysates of *Pneumococcus* by previous inoculations of the same material. More recently Bull and McKee (14) demonstrated that after recovery from infection induced by intranasal instillation of live pneumococci, rabbits become highly skin sensitive to an autolysate of pneumococci. These authors also reported that such rabbits die of shock following intravenous injection of the same material.

Skin reactions in patients suffering with pneumonia have also been reported. Clough (6) showed that pneumonia patients reacted locally when a protein extract of the organism was injected into the skin, but this reaction did not vary materially from the one occurring in normal individuals. Weil (15) described a skin reaction in patients to an autolysate of *Pneumococcus* which begins to appear at about the time of crisis. Steinfeld and Kolmer (16) reported the occurrence of a skin reaction following intracutaneous injections of heat-killed bacteria in 30 per cent of pneumonia patients after crisis. Later Weiss and Kolmer (17) showed that patients with pneumonia gave skin reactions to pneumotoxin (a sodium choleate solution of the cell) from the fifth to the thirteenth day of the disease. Bigelow (18) also obtained skin reactions in pneumonia patients with heat-killed pneumococci and several soluble derivatives. Herrold and Traut (19) found that 73 per cent of pneumonia patients failed to react to injections into the skin of a filtrate of a 5-day culture of *Pneumococcus*, while only 15 per cent of normal persons failed to react. Poole, Bumstead and Blake (21) obtained skin reactions with a protein extract of *Pneumococcus* in patients after crisis. Tillett and Francis (20) have recently demonstrated that patients with pneumonia react locally to injections of the protein-free carbohydrate of *Pneumococcus*. The reaction occurs at about the time of crisis, is type-specific, immediate, and is of the wheal and erythema variety. They also obtained delayed skin reactions in patients with the

nucleoprotein of *Pneumococcus*. Except for immediate reactions to the carbohydrate described by Tillett and Francis the skin reactions described by all these observers followed injections of whole bacteria or protein derivatives and the reactions were of the "delayed" type.

In the present paper is reported the occurrence of skin reactions in rabbits to derivatives of *Pneumococcus*. It has been found that following repeated intracutaneous injections either of heat-killed *Pneumococcus*, or the "nucleoprotein," or of the supernatant liquid after the "nucleoprotein" has been precipitated from a bacterial extract, rabbits become skin reactive. The skin reactions are of the delayed type and may be elicited by injections of "nucleoprotein," or of a solution of the cells from which both acid precipitable and heat coagulable proteins have been removed. This skin reactivity, however, is not elicited by the specific carbohydrate of *Pneumococcus*.

The skin reactivity was found to occur in rabbits following repeated injections by the intravenous or intracutaneous route of the bacteria, or the protein derivatives of *Pneumococcus*. The skin reactivity to *Pneumococcus* protein appears to be unrelated to type-specificity, or to resistance of the animal to infection. On the other hand, the occurrence of the skin reactivity appears to be associated with the presence of circulating species-specific antibodies.

#### SUMMARY AND CONCLUSIONS

1. A skin reaction elicited by the injection of the *Pneumococcus* "nucleoprotein," or of a solution of the cells from which the acid and heat-coagulable proteins have been removed is described in rabbits which have previously received repeated intracutaneous injections of heat-killed pneumococci.

2. In terms of bacterial specificity, the skin reaction is considered to be not type-specific, but species-specific.

3. A similar skin reaction to the proteins of *Pneumococcus* occurs in rabbits following the repeated administration by the intravenous or intracutaneous route of the heat-killed organisms or their protein derivatives.

4. The skin reaction may occur independently of resistance to infection.

5. The skin reaction appears to be related to the presence of circulating species-specific antibodies.

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# REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

## V. THE DEVELOPMENT OF EYE REACTIVITY TO DERIVATIVES OF PNEUMOCOCCI

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The present paper describes the development of eye reactivity to certain products of *Pneumococcus* as a result of repeated intracutaneous injections of the heat-killed bacteria. For the purpose of comparison, additional rabbits were given injections of the cells or their derivatives by other routes and the reactivity of the eyes in these animals was also studied.

### EXPERIMENTAL

*Methods.*—The injection of rabbits with *Pneumococcus* or its derivatives was carried out as described in a previous communication (1).

*Eye Test.*—Eye tests were done according to the technique described by Derick and Swift (3, 4). The cornea was anesthetized with one drop of 10 per cent novocaine, and then it was lightly scarified with a cataract knife. Three scratches were made on one of the upper quadrants of the cornea beginning 1 to 2 mm. from the sclerocorneal junction and reaching to the center. One drop of the reagent was instilled into the conjunctival sac and the material was rubbed gently over the surface of the cornea by manipulation of the lid.

Rabbits received from 8 to 12 intracutaneous injections of bacterial suspension. About 20 days after the last injection eye tests were performed by dropping into the eye, prepared as previously described, one drop of a solution of "nucleoprotein" (2). For purpose of control, two drops of the same solution were dropped into one of the eyes of each of a series of normal rabbits.

In the normal controls no changes in the eyes were observed. In certain of the animals which had previously received intracutaneous injections definite reactions occurred. These reactions were of two kinds. In the less severe type of reaction there appeared, after

several hours, a more or less extensive injection of the vessels of the palpebral and scleral conjunctivae without any change in the cornea. The maximum reactivity was attained within 24 to 48 hours and the reaction usually lasted 3 to 5 days.

In the severe type of reaction, in addition to the conjunctival injection, there developed on the first or second day a turbidity over the scarified area on the cornea. On the third or fourth day, in certain cases, small loops of blood vessels, a pannus, began to appear near the sclerocorneal margin and the vessels grew downward toward the center. Occasionally, there appeared to be hemorrhage into the cornea surrounding the newly formed blood vessels. There was frequently a certain degree of edema of the conjunctivae, and in the more severe reactions, there was present in the conjunctival sac a mucopurulent

TABLE I

*The Incidence of the Ophthalmic Reaction in Rabbits Following Repeated Intracutaneous Injections of Heat-Killed Pneumococci*

Total number tested	Number giving eye reaction			Number giving no eye reaction
	Conjunctival	Corneal		
		With pannus	Without pannus	
60	8	15	32	20

discharge which was proved to be bacteria-free by smears and cultures. The general corneal turbidity did not last so long as the pannus, and the eye appeared normal again within 7 to 12 days, the duration depending upon the severity of the reaction. It will be noted that the corneal reaction described here resembles very closely the ophthalmic reaction reported by Derick and Swift (4) in their studies on hypersensitiveness of rabbits to non-hemolytic streptococci.

In Table I, a summary has been made of the occurrence of the eye reaction to nucleoprotein in rabbits which had received repeated injections of heat-killed pneumococci. It is seen that of 60 rabbits tested, in 40 an eye reaction occurred, in 20 there were no reactions. Of the 40 animals showing an eye reaction, in 8 the reaction was limited to the conjunctivae, in 32 the cornea was involved, and in 15 of these

a pannus developed. Experiments were then conducted to determine whether the eye reactions were elicited by a solution of pneumococci from which the acid and the heat coagulable proteins had been removed. As in the preceding experiments, a series of rabbits were given repeated intracutaneous injections of solutions of heat-killed pneumococci. These animals were then tested by placing in the eye one drop of the *Pneumococcus* solution. Two drops of the solution were also placed in the eye of each of a series of control rabbits. It was found that while the eyes of normal rabbits were unaffected by this material, eye reactions occurred in treated animals, but not with so great a regularity as with "nucleoprotein."

In other rabbits the reactivity of the eye was tested by employing the soluble specific substances of *Pneumococcus*. The carbohydrates were used in dilutions of 1-1000, 1-10,000, and 1-25,000, but eye reactions were not observed in any of the animals tested with the homologous or heterologous carbohydrates.

Since Derick and Swift elicited the ophthalmic reaction described by them by the instillation into the eyes of living bacteria, experiments were conducted to determine whether the increased eye reactivity following the repeated intracutaneous injection of pneumococci could also be demonstrated by the instillation of living organisms. As the instillation of living *S* pneumococci incites an infection which may obscure the reaction, living *R* cells were employed. An *R* strain was selected which was derived from a type of *Pneumococcus* other than that used for the preparatory injections. After scarification of the cornea of each of a series of rabbits that had previously received repeated intracutaneous injections of a suspension of heat-killed pneumococci, one drop of the living culture, concentrated 25 to 30 times, was transferred into the conjunctival sac. In no instance, however, was an eye reaction observed following the instillation of living *R* pneumococci.

#### *The Rate of Development and Duration of the Eye Reactivity*

In the following experiment the rate of development of eye hypersensitiveness in rabbits previously injected intracutaneously with suspensions of heat-killed *Pneumococcus* was studied. Fourteen rabbits were given one or more injections each. The rabbits were divided



into seven groups of two each, and the animals of every pair received the same number of inoculations. The animals of the various pairs received a different number of inoculations, those of the first pair receiving 1 injection, those of the last pair 7 injections. Three weeks after the final injections, the rabbits were tested for eye reactivity to "nucleoprotein." The results of this experiment are given in Table II. It is apparent that there is no regularity between the development of the eye reactivity and the number of intracutaneous injections of bacterial suspension. Thus, both rabbits receiving only 1 inoculation of bacterial emulsion showed severe eye reactions, while the two

TABLE II

*The Development of Eye Reactivity in Rabbits Injected Intracutaneously with Heat-Killed Pneumococcus*

Number of rabbits	Number of injections	Results of eye reaction			
		Negative	Positive		
			Conjunctival	Corneal	
				With pannus	Without pannus
2	1	0	0	2	0
2	2	0	1	1	0
2	3	1	0	1	0
2	4	1	0	0	1
2	5	0	0	1	1
2	6	2	0	0	0
2	7	0	0	1	1

that received 6 injections showed no reactions; and again, eye reactions were obtained in both rabbits which had received 7 inoculations of pneumococci in the skin. This irregularity indicates that individual rabbits vary greatly in the manner in which they react to intracutaneous injections of pneumococci, especially as regards the development of eye hypersensitiveness.

In another experiment, the duration of the eye reactivity was studied. It was found that there is considerable variation in different animals. The reactivity of the eye may last as long as 4 months; in two rabbits, the eye reaction was elicited 6 months after the last intracutaneous injection of bacterial suspension.

During the course of this study, rabbits which had previously been shown to be eye sensitive but in which the eye sensitiveness had later disappeared were subsequently injected with "nucleoprotein" solution intravenously or by some other route. It was frequently noted under these conditions that although the cornea was not scarified and nothing was instilled into the eye, following the subsequent injection of the protein, the conjunctivae became congested and a typical eye reaction reappeared. The longer the interval between the disappearance of the ophthalmic reaction and the subsequent injection of protein, the less frequently did this reappearance of the reaction take place. However, in one rabbit, under these conditions an eye reaction reappeared after an interval of 6 months.

#### *The Development of Eye Reactivity Following Administration of the Intact Cell by Different Routes*

The following experiment was carried out to determine whether eye reactivity results not only when injections of heat-killed pneumococci are made into the skin but also when the injections are made by other routes. Accordingly, several rabbits of one group were given repeated intravenous injections and those of another group intraperitoneal injections. The results of this experiment are summarized in Table III. It will be seen that in rabbits injected intravenously eye reactions were never obtained. Only two rabbits were given intraperitoneal injections, and neither gave an eye reaction when tested. It is appreciated, however, that the number in this instance is too small to allow a definite conclusion. Swift and Derick (4) have also shown in the case of hypersensitiveness to nonhemolytic streptococci, that the increased eye reactivity does not result from intravenous injections.

#### *The Development of Eye Reactivity Following Repeated Injections of Soluble Derivatives of Pneumococcus*

A study was made of the incidence of the eye reactivity in rabbits that have received repeated injections of solutions of various cell constituents. Rabbits were given intracutaneous or intravenous injections of (1) a solution of the bacteria prepared by freezing and thawing, (2) a solution of the "nucleoprotein" obtained by precipita-

tion of the bacterial solution by weak acid, (3) the supernatant fluid after precipitation of the "nucleoprotein," and (4) a solution of the bacterial cells resulting from autolysis. In Table III a summary is given of the results of this experiment. It is seen that in none of a total of 41 rabbits have eye reactions been observed after the animals had received repeated intracutaneous or intravenous injections of solutions derived from pneumococci.

TABLE III

*The Incidence of the Eye Reaction in Rabbits Repeatedly Injected with Pneumococci or Their Products by Different Routes*

Material administered	Route of administration	Number of rabbits	Result of eye test	
			Positive	Negative
Heat-killed Pneumococcus (R or S)	Intracutaneous	60	40	20
	Intravenous	91	0	91
	Intraperitoneal	2	0	2
Solutions	Intravenous	17	0	17
Nucleoprotein	Intracutaneous	8	0	8
	Intravenous	8	0	8
Solution (nucleoprotein removed)	Intracutaneous	4	0	4
Autolysates	Intravenous	6	0	6

*The Development of Eye Reactivity Following Infection by  
Pneumococcus*

The question naturally arises whether eye reactivity accompanies or follows infections by *Pneumococcus*. Rabbits were infected by injections of live cultures of *Pneumococcus*. Infection was induced by three different routes.

*1. Infection by Skin.*—Each of 18 rabbits received an intracutaneous injection of 0.2 cc. of an 8 to 10 hour culture of Type III *Pneumococcus*. All the animals developed a septicemia lasting from 1 to 7 days, and all but 2 eventually recovered from the infection. Viable organisms were recovered from the skin lesion only

during the first 2 to 4 days. About 3 weeks after infection had been induced, the animals were tested by the instillation into the conjunctival sac of "nucleoprotein" derived from a strain of *Pneumococcus* Type II. Five of the 16 rabbits gave positive eye reactions.

2. *Infection by the Trachea*.—Twelve rabbits received varying amounts of young broth cultures introduced intratracheally. Temperature variation in these animals indicated that infection occurred. All recovered. Sixteen days later eye tests were conducted in the usual manner. Of the 12 rabbits, 4 gave the ophthalmic reaction when tested in the usual way.

3. *Infection by Spraying*.—Seventeen rabbits survived infection by spraying with pneumococci according to the technique described by Stillman (5). These animals were tested for the presence of eye reactivity 2 to 3 weeks after the spraying. Of the 17 rabbits, 5 gave positive eye reactions. The data of the experiment are given in Table IV.

TABLE IV

*The Incidence of the Eye Reaction in Rabbits Following Artificial Infection with Pneumococcus*

Method of infection	Number of rabbits	Eye reaction	
		Positive	Negative
Skin.....	16	5	11
Intratracheal.....	12	4	8
Spraying.....	17	5	12

It is evident that eye reactions may be elicited in rabbits following experimental infection by *Pneumococcus* in the skin or trachea.

*Relation between Eye Reactivity and Active Resistance to Infection*

It has been pointed out in an earlier communication (6) that rabbits acquire an active resistance to infection following repeated intracutaneous injections of heat-killed pneumococci. It is now seen that rabbits so injected also may develop an eye reactivity to derivatives of *Pneumococcus*. On the other hand, rabbits receiving intravenous injections also acquire a resistance to infection, but they do not become eye reactive. Moreover, rabbits which have been given repeated injections of solutions derived from *Pneumococcus* acquire neither a resistance to infection (6), nor eye reactivity. While it may be concluded, therefore, that eye reactions do not necessarily accompany

active resistance to infection it is important to note that eye reactions were never observed in animals without resistance to infection.

*Relation of Eye Reactivity to the Secondary Reaction*

It has been previously reported (7) that the reaction at the site of injection following the first intracutaneous inoculation of heat-killed pneumococci in a rabbit usually disappears within 4 or 5 days. At about the tenth day, however, in certain animals there occurs a recrudescence. It seemed of interest to determine if possible whether the animals showing a secondary reaction were especially prone to develop eye hypersensitiveness. The data bearing on this point are summarized in Table V. It will be seen that a total of 23 rabbits were observed. Of 15 animals developing a secondary reaction, 12

TABLE V

*Relation of the Ophthalmic Reaction to Secondary Skin Reaction*

Total number of rabbits	Rabbits giving eye reaction	Rabbits not giving eye reaction
With secondary skin reaction, 15.....	12	3
Without secondary skin reaction, 8.....	3	5

gave a positive ophthalmic reaction, while 3 did not. Of 8 rabbits showing no secondary reaction, only 3 gave positive eye reactions. Although there is no absolute correlation between the development of eye sensitivity and the secondary reaction, nevertheless, eye reactions appear to occur with greater frequency in the rabbits which show the secondary reaction. Derick and Swift (4) also report the existence of a "rough parallelism" between the occurrence of eye reactions and the secondary reactions in rabbits injected with *Streptococcus viridans*.

DISCUSSION

The present communication records the development of an increased eye reactivity in certain rabbits which have previously received repeated intracutaneous injections of heat-killed pneumococci. The eye reactivity is manifested by a reaction, after scarification of the

cornea, to either the "nucleoprotein" or a solution of *Pneumococcus* from which the acid precipitable and heat coagulable proteins have been removed. The reaction, in the less severe form, consists of the injection of the conjunctival blood vessels; in the more severe forms, the cornea is also involved and exhibits a turbidity with frequently the formation of a pannus. Living R cells or the specific soluble substances of *Pneumococcus* do not elicit the eye reaction.

Unlike the skin reactivity reported previously, the eye reactivity does not occur in rabbits which have been given repeated intravenous injections of the heat-killed bacteria. Eye reactivity does not follow repeated intravenous or intracutaneous injections of rabbits with various solutions derived from the organism.

Increased eye reactivity was also found to be present in animals which had recovered from infection induced by injection of pneumococci into the skin or trachea, or by spraying. The eye reaction was elicited only in animals resistant to infection, but the relationship of the eye reactivity to active resistance still remains obscure. The eye reaction occurred more frequently in rabbits with a secondary reaction following the primary skin reaction to the first intracutaneous injection of bacteria than it did in animals not exhibiting a secondary reaction.

It may be of interest to point out that Clough (9) called attention to the "ophthalmo-reaction" in pneumonia patients. Without scarifying the cornea, he observed in 6 of 15 patients an eye reaction to one drop of 1 per cent *Pneumococcus* protein, while only 1 of 20 normal individuals gave a similar reaction.

#### SUMMARY AND CONCLUSIONS

1. About two-thirds of the rabbits injected intracutaneously with suspensions of heat-killed S or R pneumococci develop an increased eye reactivity.

2. Eye reactions in these animals may be elicited by the instillation of "nucleoprotein," or of a solution of *Pneumococcus* from which the acid precipitable and heat coagulable proteins have been removed. The eye reactions are not elicited, under the conditions described, by living R cells or the protein-free, type-specific, polysaccharides.

3. Rabbits do not develop an increased eye reactivity following intravenous injections of the intact cell.

4. Rabbits do not develop an increased eye reactivity following injections of soluble derivatives of *Pneumococcus*.

5. Experimental infection by *Pneumococcus* may stimulate eye reactivity in rabbits.

6. Eye reactivity occurs in animals actively resistant to infection.

7. Eye reactions are observed more frequently in rabbits which show the secondary reaction.

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# REACTIONS OF RABBITS TO INTRACUTANEOUS INJECTIONS OF PNEUMOCOCCI AND THEIR PRODUCTS

## VI. HYPERSENSITIVENESS TO PNEUMOCOCCI AND THEIR PRODUCTS

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### PLATE 11

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In the preceding papers of this series, it has been shown that when rabbits are injected intracutaneously with a suspension of heat-killed pneumococci, a reaction occurs at the site of injection (1). With repeated injections, the reactions increase in size and intensity until four to six inoculations have been made, after which the reactions become decreased. Following the intracutaneous injections, the rabbits acquire a skin reactivity to certain derivatives of *Pneumococcus*, and in certain instances, also an eye reactivity to the same derivatives (2, 3). It was pointed out that while a state of skin reactivity may follow the injection of solutions of pneumococci as well as suspensions of the dead organisms and regardless of the route of administration, the state of eye reactivity arises only when intact cells have been injected intracutaneously, and less frequently following experimentally induced infection with *Pneumococcus*. Under either condition, it seems that a localization of the bacteria is essential in order that the animals develop eye reactivity.

It is well known that when rabbits that have received repeated injections of non-bacterial protein, and are later injected with small amounts of the same protein into the skin, marked reactions occur, the "Arthus reaction" (4). This skin sensitivity is now definitely known to be associated with the presence of specific antibodies in the circulating blood. It seems likely that the "skin reactivity" to pneumococci, and their protein derivatives, closely resembles the Arthus reaction, while the "eye reactivity" seems to depend upon



factors which are different from those operative in the phenomenon of "skin reactivity." It has seemed advisable therefore to compare all these reactions in a series of rabbits treated simultaneously.

### EXPERIMENTAL

Each rabbit of three groups of eight received repeated injections of one of three different preparations. All the rabbits of the first group received injections of a solution of crystalline egg albumin; each of the second group received injections of a solution of "nucleoprotein" derived from a Type II (S) *Pneumococcus*; the rabbits of the third group received injections of a suspension of heat-killed Type I *Pneumococcus*. In four animals of each group the injections were made intracutaneously, in the other four the injections were made intravenously.

The injections were made once a week in quantities of 0.2 cc., which, in the case of the bacterial suspension, contained the organisms from 2 cc. of the original broth culture; while in the case of the protein solutions, 0.2 cc. of the one solution contained an amount of "nucleoprotein" of *Pneumococcus* estimated to be 0.6 mg., and 0.2 cc. of the egg albumin solution contained 3 mg. of the protein. The number of injections differed in the different animals, as will be brought out later. The animals were bled immediately preceding each injection, so that the development of circulating antibodies might be followed. Following each injection the character of the reaction was carefully recorded. At definite intervals, to be specified later, eye and skin tests were performed by the technique described in former communications (2, 3).

#### *Repeated Injections of Rabbits with Crystalline Egg Albumin*

*a. Intracutaneous Injections.*—Two rabbits were given 5 weekly injections of egg albumin in the skin and two animals received 10 similar injections. The earlier intracutaneous injections of egg albumin were not followed by definite local reactions. With later injections, there was always an extensive reaction at the site of injection. Thus following the first injections, only a pale erythematous blush was seen, and this disappeared within a day or two. In two rabbits, the fourth injection into the skin caused a definite reaction, which consisted of a central area of ecchymosis surrounded by a broad area of edema. The fifth injection was followed by a local reaction in all the rabbits. This reaction was typical of the Arthus phenomenon as already described by a number of workers (Arthus (4), Nicolle (5), Lewis (6), Opie (7), and others). The reaction following the sixth injection was perhaps more severe, but with subsequent injections the intensity of the skin reaction remained approximately the same. The more severe reactions began to appear within a few hours after the injection, and reached a maximum intensity in from 24 to 48 hours. At that time (Fig. 2) the size of the reactions varied from 4 to 6 cm. in diameter; they were circular, circumscribed and markedly elevated. The reactions contained a central area of ecchymosis and

necrosis which measured from 1 to 2.8 cm. The ecchymosis was surrounded by a thin, crimson band, 0.2 to 0.5 cm. wide. The remainder of the lesion was pink to red in color and consisted of a soft edematous ring. The edema usually disappeared within 3 to 5 days, and the area of ecchymosis was gradually transformed within a week into a black scab which persisted for 2 weeks or more.

A study of the precipitating antibodies present in the sera of these rabbits each week revealed that the precipitins increased gradually, the titre of the serum reaching 1:400,000 by the fifth injection. The titre continued to increase until the seventh injection when a maximum of 1:1,000,000 was reached, and it was maintained at that point during the time the injections were continued. The antibody content of the serum, therefore, rose concomitantly with the reactivity of the skin.

Twenty days after the final intracutaneous injection of egg albumin, the rabbits were tested for skin and eye hypersensitiveness. Skin tests were conducted by injecting into the skin different quantities of egg albumin. The dilutions employed contained from 3 to 0.015 mg., and the volume of fluid injected in each test was 0.2 cc. Skin tests were also performed on each rabbit with 0.2 cc. (0.6 mg.) of the *Pneumococcus* "nucleoprotein." There was some variation in the severity of the reactions to the smaller quantities of egg albumin, but the animals reacted locally with all the dilutions employed. The reactions were of the same character as those described above. No skin reactions were observed following the intracutaneous introduction of *Pneumococcus* protein.

Eye tests were done on the same day by the technique used in the preceding study (3). After scarifying the cornea, one drop of a solution of egg albumin was instilled into the conjunctival sac. The opposite eye was tested in a similar manner by instilling one drop of a *Pneumococcus* "nucleoprotein" solution. In none of the rabbits of this entire group were eye reactions elicited either by the solution of egg albumin or by the solution of "nucleoprotein" of *Pneumococcus*.

*b. Intravenous Injections.*—Four rabbits were given each week an intravenous injection of 0.2 cc. of a solution of egg albumin containing 3 mg. of the protein. Two rabbits received a total of 5 injections and two animals were given 10 inoculations.

The development of precipitating antibodies in the sera of these rabbits was similar to that observed in the rabbits which were given intracutaneous injections. The precipitin titre of the sera gradually increased to 1:1,000,000 at the seventh injection, and it remained at this point during the period of subsequent injections.

Twenty days after the last intravenous injection of egg albumin the animals were tested for skin and eye hypersensitiveness. The tests consisted of the inoculation of solutions of egg albumin and of *Pneumococcus* "nucleoprotein" into the skin and the separate instillation of these solutions into the conjunctival sacs in the same manner as described in testing the animals injected intracutaneously. Typical Arthus reactions in the skin were obtained in all the rabbits at the site of injection of the egg albumin solution, while no skin reactions occurred at the site

of injection of the *Pneumococcus* "nucleoprotein" solution. No eye reactions occurred in any of the animals tested by the instillation of either the egg albumin or the "nucleoprotein" solution. Derick and Swift (6) and Rhoads (9), also found that eye sensitivity did not occur in rabbits which gave typical Arthus reactions in the skin.

*Repeated Injections of Rabbits with the "Nucleoprotein" of Pneumococcus*

Eight rabbits were given repeated injections of a solution of "nucleoprotein" derived from a strain of Type II *Pneumococcus*. Injections of 0.2 cc. of the solution were made once a week. In four of the rabbits the injections were made intracutaneously and in the remainder, intravenously.

*a. Intracutaneous Injections.*—Of the four rabbits receiving intracutaneous inoculations, two received 5 injections and two, 10 injections. Following the earlier injections no visible local reactions occurred. Following the fourth injection all of the animals exhibited a reaction at the site of injection. At this time the reactions measured from 4 to 6 cm. in diameter. The skin showed slight, if any, elevation, but it was definitely thickened, and it assumed a pink color. With subsequent injections, the reactions increased in intensity until the seventh inoculation, and from then on the reactions remained of about the same intensity. The more severe reactions measured from 5 to 8 cm. in diameter. The skin was definitely elevated and was of a deep red color. A central area of ecchymosis frequently developed, but necrosis of the skin was never observed. The edema usually disappeared in 2 or 3 days, and within 5 to 6 days the lesion was completely resolved. These skin reactions to the "nucleoprotein" of *Pneumococcus* were strikingly similar to the Arthus reactions observed in rabbits hypersensitive to egg albumin, but they were never as severe as the latter reactions. This resemblance is seen in Figs. 1 and 2, which illustrate both skin reactions.

The development of circulating antibodies was estimated by determining each week the ability of the sera of these rabbits to cause agglutination of R pneumococci. A gradual increase in the agglutination titre occurred until the seventh injection, when a titre of 1:320 was reached, and the titre was maintained at that point during the remaining period of injection.

Twenty days after the last injection tests of skin and eye hypersensitiveness were made. The sensitivity of the skin was tested by the injection of 0.6 mg. (0.2 cc.) of the nucleoprotein of *Pneumococcus* and 3 mg. (0.2 cc.) of egg albumin. Eye tests were also performed with these proteins. In all the rabbits, skin reactions of the type already described occurred at the site of injection of the *Pneumococcus* "nucleoprotein" only. No reactions occurred at the site of injection of the solution of egg albumin. None of the rabbits exhibited eye reactions to either protein.

*b. Intravenous Injections.*—Four rabbits were given intravenous injections of a solution of *Pneumococcus* "nucleoprotein." Two rabbits received 5 injections and two received 10 injections. The development of antibodies in the sera of these

animals was studied, and it was found that the rate and time of appearance of anti-R agglutinins in these rabbits did not vary materially from the rate and time of appearance of those antibodies in the rabbits receiving intracutaneous injections of *Pneumococcus* "nucleoprotein" solution. The subsequent injections had no further effect on the antibody titre of the sera.

Twenty days after the last injection of the antigen, the animals were tested for skin hypersensitiveness by intracutaneous injections of solutions of *Pneumococcus* "nucleoprotein" and of egg albumin, and they were tested for eye hypersensitiveness by the separate instillation of solutions of these two proteins into the conjunctival sacs. The same quantities of the solution and the same methods were used as those employed in the previous experiment. Skin reactions occurred in all animals at the site of injection of the *Pneumococcus* "nucleoprotein." No skin reactions occurred at the site of injection of the egg albumin solution. The positive skin reactions resembled those which occurred in the animals which had previously been given repeated injections of *Pneumococcus* protein into the skin. No eye reactions occurred in any of the animals following the instillation of either the "nucleoprotein" solutions or the egg albumin solution.

### *Injection of Rabbits with Suspensions of Heat-Killed Pneumococcus Type I*

Eight rabbits were given repeated weekly injections of suspensions of heat-killed *Pneumococcus* Type I; in four the injections were made into the skin and in four into a vein. At each injection 0.2 cc. of the suspension was given and this contained the bacteria from 2 cc. of broth culture.

*a. Intracutaneous Injections.*—Two rabbits received 5 injections and two received 10 injections. The reaction of the skin to successive injections of pneumococci has been described in detail in an earlier communication (1), but for purposes of comparison with the skin reactions to soluble proteins, however, they will be described again in brief. The first injection was followed locally by a sharply circumscribed, indurated, pink nodule which disappeared within a few days. A secondary reaction frequently followed the disappearance of the first reaction. With repeated inoculations, the reaction at the site of injection increased gradually in size, intensity, and duration until the fourth to the sixth injection. At this time, the skin was markedly elevated and assumed a reddish to purple hue. There was considerable edema and a central area of ecchymosis appeared at the site of which the skin frequently breaks down. The lesion of the severe reaction persisted for 3 to 5 weeks. The reaction following the injections after the fourth to the sixth had a modified character; they were flat, without edema or ecchymosis; they varied considerably in size, and they disappeared comparatively rapidly.

Weekly tests of the sera of these animals failed to show the presence of type-specific antibodies at any time and none of the sera possessed protective power for mice. Species-specific antibodies were detected as anti-R agglutinins for the first

time at the third bleeding. After this the titre of the serum for these agglutinins increased gradually and after the seventh injection an agglutination titre of 1:1280 was reached. The titre was maintained at that point during the remainder of the period of injections. Twenty days after the last injection of bacterial suspension these animals were tested for skin and eye hypersensitiveness. The injection of a solution of the "nucleoprotein" of *Pneumococcus* into the skin gave rise to a reaction at the site of injection in all animals and these reactions resembled the skin reaction to the "nucleoprotein" in the animals which had previously received repeated injections of the "nucleoprotein" solution. Reactions occurred in the eyes of three of the rabbits following the instillation of the solution of "nucleoprotein." In one animal no reaction occurred. The eye reactions involved the cornea but were without a pannus. (See Figs. 3 and 4.) These reactions have been described fully in a previous paper (3).

*b. Intravenous Injections.*—Four rabbits were given intravenous injections of heat-killed pneumococci Type I. The schedule of injections was the same as that employed for the intracutaneous inoculations. Two rabbits received 5 injections and two, 10 injections.

A study of the presence of antibodies in the sera of these rabbits showed that type-specific agglutinins were demonstrable after the second injection in a titre of 1:5 and 1:10. These antibodies increased and after the fourth injection the agglutination titre of the serum was 1:80. The titre was not influenced by further injections of the bacterial emulsion. Species-specific antibodies (R agglutinins) also appeared in the serum but the increase was more gradual. After the third injection, the titre of the serum for these antibodies was 1:10, and it increased to 1:640 after the fifth injection. From then on, the titre fluctuated slightly above and below that point.

Twenty days after the last injection of bacteria tests for skin and eye hypersensitiveness were conducted as described above. Injections into the skin of a solution of "nucleoprotein" of *Pneumococcus* gave rise to reactions at the site of injection in all the rabbits. These reactions could not be differentiated from those observed in the animals which were tested 20 days after repeated intracutaneous injections. Tests for eye hypersensitiveness all gave negative results.

The observations made in these experiments, in which the reactions occurring in three groups of rabbits treated in various ways were studied, may be summarized as follows:

With repeated intracutaneous injections of egg albumin, "nucleoprotein" of *Pneumococcus* or heat-killed *Pneumococcus* cells the rabbits all showed reactions at the site of injection. After each of the first 3 injections of egg albumin there occurred a faint erythematous blush. The reaction following the fourth injection was more severe, while the reactions following the fifth and sixth injections were very

severe, with necrosis of the skin and scab formation, the typical Arthus phenomenon. The reactions following the subsequent injections were approximately of the same intensity as those following the fifth and sixth injections.

When, instead of a solution of egg albumin, a solution of the "nucleoprotein" of *Pneumococcus* was repeatedly injected into the skin no reactions occurred after the first 3 injections. After the fourth injection a mild reaction occurred and after the fifth, sixth and seventh injections reactions of a considerable grade of severity were seen. These reactions also resembled the Arthus phenomenon, except that they were less severe. No necrosis of the skin was ever observed. The local reactions after the subsequent injections were of the same character and of the same intensity as those after the fifth and sixth injections.

When suspensions of bacterial cells were repeatedly injected into the skin, reactions at the site of injection occurred after the first and all subsequent injections. The reaction after the first injection was not of great severity and disappeared within 4 to 5 days. On about the tenth day, however, there occurred an exacerbation of the lesion at the same site, the so-called "secondary reaction." The intensity of the reactions which occurred following the subsequent injections became greater up to the fourth and sixth injections, after which the reactions became increasingly milder. No secondary reactions occurred except following the first injections. The superficial aspect of the skin reactions following the repeated injections of the suspensions of bacterial cells did not differ essentially from those of the reactions after the injections of egg albumin and "nucleoprotein" solutions, though in the former there has seemed less tendency for a zonal arrangement to be present, with ecchymosis in the center, about this erythema, and surrounding this an area of edema. But too much stress must not be laid on the difference in the superficial appearance of the different reactions. In the severe reactions after injection of suspensions of whole cells, necrosis and scab formation sometimes occurred just as they did after injections of egg albumin.

The reactions following the injections of whole cells do differ, however, from the reactions following repeated injections of egg albumin or "nucleoprotein" in that with the former every injection.

including the first, is followed by a reaction, while with the latter the reactions do not occur until 2 or 3 injections have been made. Moreover, following injections of egg albumin, or bacterial "nucleoprotein" when the reactions have become of maximum intensity, the reactions to all subsequent injections are also of maximum grade. On the other hand, when a reaction of maximum degree has followed one of the injections of suspensions of whole cells, the reactions to subsequent injections are milder and of a somewhat different character.

While, therefore, the skin reactions following injections of solutions of egg albumin, or bacterial "nucleoprotein," or of suspensions of pneumococcal cells, all resemble one another superficially and are like the Arthus phenomenon, the reactions following injections of the whole cell have certain characteristics, and others will be mentioned later, which indicate that they are of a different character. When the rabbits, which had received repeated weekly injections either intravenously or intracutaneously, of solutions of egg albumin, or solutions of *Pneumococcus* "nucleoprotein," or suspensions of heat-killed pneumococci, were tested 20 days after the last injection by injecting 0.2 cc. of a solution of egg albumin, or bacterial "nucleoprotein" intracutaneously, local skin reactions occurred in certain animals. In the animals which had been previously repeatedly injected intravenously or intracutaneously with egg albumin, typical Arthus reactions occurred when egg albumin solution was injected, but no reaction occurred when "nucleoprotein" solution was injected. Exactly the opposite results were obtained in the animals which had previously received injections of a solution of *Pneumococcus* "nucleoprotein." The reactions were therefore quite specific as concerns the proteins employed. In all cases these skin reactions were of a mild "Arthus" type. In the animals previously repeatedly injected, with suspensions of heat-killed pneumococci, intravenously or intracutaneously, no skin reactions were observed following the subsequent injection of egg albumin. When the tests were made with "nucleoprotein" solutions, however, definite skin reactions occurred and these did not differ materially from the reactions occurring in the tests previously mentioned.

Eye tests were performed on all the animals 20 days after the preparatory injections had been given. Into one eye a solution of egg albumin was instilled and into the other eye a solution of "nucleo-

protein" was instilled. In no cases were reactions observed except in animals in which previous repeated intracutaneous injections of a suspension of heat-killed pneumococci had been made. In three of these animals typical eye reactions (3) occurred, in one animal no change was observed.

The time of occurrence of all these reactions in the skin and eye was studied with especial reference to the development of antibodies in the blood. It will be seen by referring to the description of the experiment, that during the period when repeated injections of egg albumin and "nucleoprotein" were being made, either intracutaneously or intravenously, antibodies specific for the respective proteins gradually developed in the blood. They reached a maximum concentration about the seventh injection, and the titre of antibodies in the blood then remained at the maximum level during the remainder of the period of observation. The antibodies appearing in the serum coincident with the injections of *Pneumococcus* "nucleoprotein" were of the species-specific kind.

It will be observed that in the animals receiving repeated injections of egg albumin and "nucleoprotein" the development of antibodies in the blood ran a course parallel to the occurrence of the skin reactions following the repeated injections of the proteins. The concentration of antibodies continued at a high level and was high at a time when the subsequent skin tests were made, 20 days after the end of the series of injections. There seems therefore to be a definite relationship between the presence of antibodies in the blood and the occurrence of the skin reactions following injections of egg albumin and "nucleoprotein." This is also the case in the rabbits in which repeated intravenous injections of suspensions of heat-killed pneumococci were made. In this case type-specific as well as species-specific antibodies appeared in the blood. On the other hand, in the animals in which repeated intracutaneous injections of heat-killed pneumococci were made, only species-specific antibodies appeared in the blood, and the relation of the concentration of these antibodies in the blood to the occurrence of skin and eye reactions was not so definite. Antibodies did not appear in the blood until after the third injection and the maximum titre of the serum was not reached until after the seventh injection, following which the antibody concentration remained at the maximum level. Reactions in the skin,



however, occurred after the very first injections when no antibodies were present in the blood, and reached their maximum intensity after the fourth to sixth injections, before the concentration of antibodies in the serum had reached the maximum, and, in some cases, when the concentration of antibodies in the serum was still low. Moreover, the reactions following the later injections were modified in character and decreased in intensity and this at a time when the concentration of antibodies in the blood was still high.

The results of this experiment indicate in a more striking manner than do the previous studies that the skin and eye reactions which are observed following the intracutaneous injections of suspensions of whole cells are the manifestations of a different mechanism from that which is operative in the occurrence of skin reactions following the repeated injections of soluble proteins, or following the injection of the whole cells into the circulation. In the latter instance the reactions are associated with the presence of antibodies in the blood and are analogous to the Arthus phenomenon; in the former case the type of skin and eye reactivity depends on some mechanism directly related to the localization of the bacteria in the skin, but the exact nature of this mechanism is still obscure.

This conclusion receives additional support from experiments undertaken to test the possibility of transferring the heightened "skin and eye reactivity" from animals which have received repeated injections of protein or bacteria to previously untreated animals.

That the Arthus variety of hypersensitiveness may be transferred to normal animals was first shown by Nicolle (5). This was more recently accomplished by Opie (6) who showed, in addition, that the severity of the skin reaction to the specific antigen, in rabbits injected with sera from actively sensitized rabbits, was approximately proportional to the concentration of antibodies present in the blood of the recipient animal after the transfer.

#### EXPERIMENTAL

Previously untreated rabbits were given intravenous injections of serum obtained from a single actively sensitized animal. The quantity of serum injected varied from 15 to 30 cc. On the day following the transfer of serum the recipient animals were bled in order to determine the titre of circulating antibodies and were then tested for the presence of skin and eye hypersensitiveness.

*Transference of Hypersensitiveness Induced by Repeated Injections of Egg Albumin*

Each of six normal rabbits was injected intravenously with 16 to 27 cc. of serum from one of six animals which gave intense reactions when a solution of egg albumin was injected into the skin. The donors had been injected repeatedly with egg albumin, three intravenously and three intracutaneously. On the following day the precipitin titre of the sera of the recipients varied from 1:10,000 to 1:50,000. Skin tests were performed in the usual manner. Five rabbits gave distinct skin reactions, with edema and, in one of the 5 reactions, a central zone of ecchymosis was present. In the sixth animal the skin reaction was doubtful, consisting only of a diffuse erythema. Eye tests were also done, but in each instance no reaction was obtained. Control of the specificity of the skin reaction was accomplished by intracutaneous injections of *Pneumococcus nucleoprotein*. No reactions followed the administration of this material.

*Transference of Hypersensitiveness Induced by Repeated Injections of *Pneumococcus* "Nucleoprotein"*

Each of eight normal rabbits was injected intravenously with 15 to 25 cc. of serum obtained from one of eight rabbits which had received injections of the "nucleoprotein" of *Pneumococcus*, four intravenously and four intracutaneously. On the following day the species-specific antibody titre of the serum of each of the recipients was measured by agglutination of R pneumococci. The titre was found to vary in the different animals from 1:20 to 1:80. The recipient animals were tested in the usual manner for eye and skin sensitivity to *Pneumococcus* "nucleoprotein." In six of the animals more or less intense skin reactions occurred, in one a doubtful reaction, and in the remaining animal no reaction was observed. The eye reactions were negative in each animal. Control of specificity was accomplished by skin and eye tests with a solution of egg albumin and no reactions were obtained to this material.

*Transference of Hypersensitiveness Induced by Repeated Injections of Intact *Pneumococci**

Each of twenty rabbits was injected with 15 to 30 cc. of serum from one of twenty rabbits which had received repeated injections of a suspension of heat-killed pneumococci. Five of the donors received intravenous injections of S cells, and fifteen received intracutaneous inoculations of S pneumococci. It should be stated that eye sensitivity had been demonstrated in all of the donors which had received intracutaneous injections. On the following day in none of the recipient animals were anti-S agglutinins present in the sera, while the anti-R titre of the serum varied from 1:20 to 1:320. Skin tests were performed in the usual manner and all but one gave distinct and unmistakable reactions. Eye tests were done by

instillation of nucleoprotein in the conjunctival sac after scarification of the cornea. No eye reaction was elicited in any of the animals. This was to be expected in the recipient animals which received serum from donors which did not them-

TABLE I  
*Reactions of Hypersensitiveness in Rabbits*

Reactions	Following repeated injections of egg albumin		Following repeated injections of Pneumococcus protein		Following repeated injections of heat-killed pneumococci (Type I)	
	Intracutaneous	Intravenous	Intracutaneous	Intravenous	Intracutaneous	Intravenous
Reaction at site of each injection	1st 3 injec. — 4th–10th injec. +	×	1st 3 injec. — 4th–10th injec. +	×	1st injec. + increase in intensity until 4th–6th injec. Then decrease in reaction	×
Development of circulating antibodies						
(a) type-specific...	×	×	—	—	—	+
(b) species specific.	+	+	+	+	+	+
Active resistance.....	×	×	—	—	+	+
Increased sensitivity						
(a) skin.....	+	+	+	+	+	+
(b) eye.....	—	—	—	—	+	—
Transfer of sensitivity						
(a) skin.....	+	+	+	+	+	+
(b) eye.....	—	—	—	—	—	—

+ indicates presence of reaction.

— indicates absence of reaction.

× indicates tests were not done.

selves exhibit eye sensitivity, but the lack of eye reactivity in the animals which had received intracutaneous injections and which had been shown to be "eye reactive" indicates that in these animals a special type of sensitivity was present, a type in which the sensitivity cannot be transferred by serum transfusion.

## RÉSUMÉ

From the series of studies concerning the reactions of rabbits to injections of pneumococci and their products it appears that when heat-killed pneumococci or their products are repeatedly injected into rabbits, intravenously or intracutaneously, the rabbits become hypersensitive to subsequent intracutaneous injections of *Pneumococcus* proteins, as shown by a reaction in the skin, which is analogous to the Arthus phenomenon as exemplified in rabbits following injections of non-bacterial protein. The development of this hypersensitiveness occurs simultaneously with the development of species-specific antibodies in the blood, and this type of hypersensitiveness may be transferred to normal rabbits following the transfer of blood serum from the sensitive animals. The development of this type of sensitivity seems to bear no relation to active immunity, since animals repeatedly injected with soluble products of the pneumococcus cells show no active immunity and nevertheless are skin reactive.

When the whole, killed bacteria are injected repeatedly intracutaneously another type of hypersensitiveness, in addition to that just described, makes its appearance. This is shown by the fact that animals treated in this way react in a different manner to the individual injections of the bacteria into the skin. At the first injection there occurs a reaction in the skin, a primary reaction, which disappears after a few days, but is followed in about 10 days by a recrudescence. This recrudescence occurs even without a second injection, and is probably an evidence of the development of hypersensitiveness.

With the repeated intracutaneous injections of the whole organisms the skin reaction becomes gradually more severe; secondary reactions occur, however, only after the first injection. After 4 to 6 skin injections have been made the reactions become of lessened severity and are also modified in their character. The skin reactions during the course of injections, therefore, are probably different from unmodified Arthus reactions, though the Arthus type of sensitivity is coincidentally present in the animals, as is shown by their exhibiting typical Arthus reactions when they are injected with small amounts of *Pneumococcus* protein 20 days later. That another type of sensitivity develops in these animals is also shown by the fact that they become eye sensitive

to *Pneumococcus* protein, while animals treated in other ways never do. Apparently when bacteria are localized in the skin there occurs the development of a new and different kind of hypersensitiveness.

#### SUMMARY AND CONCLUSIONS

1. Rabbits receiving either intracutaneous or intravenous injection of crystalline egg albumin acquire a "skin sensitivity" but not an "eye sensitivity" to the albumin.

2. Rabbits receiving either intracutaneous or intravenous injection of the "nucleoprotein" of *Pneumococcus* acquire a specific "skin sensitivity" to the "nucleoprotein" but not an "eye sensitivity."

3. Rabbits receiving intravenous injections of a suspension of heat-killed pneumococci acquire a "skin hypersensitiveness" but not an "eye hypersensitiveness" to the "nucleoprotein."

4. The skin hypersensitiveness to the respective proteins is associated with the presence of antibodies in the blood and is transferable by the injection of serum from an actively sensitized to a normal rabbit.

5. Rabbits receiving intracutaneous injections of a suspension of heat-killed pneumococci also acquire a "skin hypersensitiveness" to the "nucleoprotein" of the cell, but they may acquire an eye hypersensitiveness as well.

6. Rabbits injected intracutaneously with a suspension of heat-killed pneumococci show a primary and a secondary skin reaction following the first injection. The intensity of the reactions increases with subsequent injections up to the fourth to the sixth injection. With later injections these reactions change in character and decrease in intensity. These reactions bear no apparent relation to the presence of antibodies in the blood.

7. Rabbits injected intracutaneously with a suspension of heat-killed pneumococci develop "eye hypersensitiveness" which, under the experimental conditions, is not transferable from "eye reactive" to normal rabbits.

8. It appears, therefore, that following the injection of heat-killed pneumococci into the skin a special kind of "eye" and "skin hypersensitiveness" develops which is not related to the presence of circulating antibodies and which cannot be transferred from the sensitive to normal rabbits.

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## EXPLANATION OF PLATE 11

FIG. 1. Cutaneous reaction in a rabbit 24 hours after the injection of 0.6 mg. of the nucleoprotein of *Pneumococcus*. The rabbit had received preliminary injections of heat-killed pneumococci intravenously, and the last injection was made 20 days before the skin test.

FIG. 2. Cutaneous reaction in a rabbit 24 hours after the injection of 3 mg. of crystalline egg albumin. The rabbit had received preliminary injections of egg albumin intravenously, and the last injection was made 20 days before the skin test.

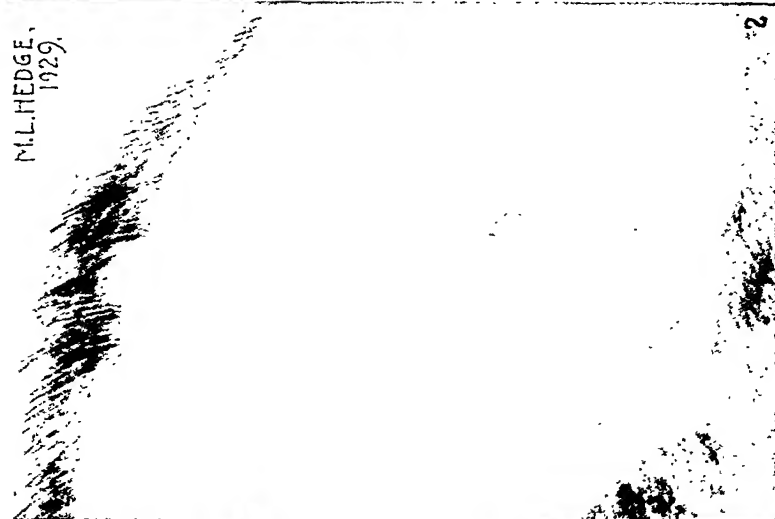
FIG. 3. Ophthalmic reaction in a rabbit which had been made hypersensitive by injections of heat-killed pneumococci into the skin. This illustrates the conjunctival reaction.

FIG. 4. Ophthalmic reaction in a rabbit which had been made hypersensitive by injections of heat-killed pneumococci into the skin. This illustrates the corneal reaction with the formation of a pannus.





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M.L. HEDGE,  
1929.





# STUDIES ON LEUKEMIA IN MICE\*

## I. THE EXPERIMENTAL TRANSMISSION OF LEUKEMIA

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In a strain of mice designated hereafter as C58, which has been inbred by brother-sister matings since 1921, it was observed that a considerable number of animals that lived more than 8 months had lymphatic leukemia.\*\* The present cooperative investigation was undertaken early in 1928, since which time the mice have regularly been autopsied and the lesions examined microscopically.

In a preliminary report (1), we recorded the existence of this leukemic strain, and the fact that the leukemia is transmissible to other mice of the same strain by inoculation with tissue emulsions, at an earlier age than that at which leukemia occurs spontaneously.

### LITERATURE

A complete account of the recorded attempts to produce or to reproduce the lesions of leukemia is not within the scope of this paper. Briefly, it may be stated that attempts to transmit leukemia from man to man, from man to lower animals, or from animals of one species to those of another, have been unsuccessful. These experiments have been reviewed recently by Opie (2).

The first and most widely known transmissible leukemia is that which occurs spontaneously in the fowl, and which Ellermann and Bang (3) and later Hirschfeld and Jacoby (4) and Schmeisser (5) transmitted to other fowl by inoculation with organ emulsions. Ellermann and Bang also transmitted the disease by inoculation

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\*\* The diagnosis was first made by Dr. Alvin M. Pappenheimer in 1926.

with cell free filtrates. Both lymphoid and myeloid leukemia were thus transmitted, and change of type was noted in some of Ellermann's experiments. Fowl paralysis (neurolymphomatosis gallinarum), although not identical with leukemia, has points in common, and is transmissible (6).

The first report of successful transmission of mammalian leukemia is that of Snijders (7), who inoculated guinea pigs with tissue emulsions of a guinea pig which had spontaneous leukemia. The lesions produced were comparable to those of the spontaneous case, and were transmissible by subsequent transfers. Not only were typical leukemias encountered, but also cases of aleukemic lymphadenosis, leukosarcomatosis, and several almost pure tumors. Snijders considered his results as evidence favoring the neoplastic theory of leukemia. Mettam (8) has found conditions resembling leukemia in cattle (East coast fever and snot-siekte) to be transmissible.

In mice both lymphoid and myeloid leukemia have been observed to occur spontaneously, notably by Tyzzer (9), Haaland (10), Levaditi (11), Slye (12), Simonds (13), and Hill (14). Tyzzer and Haaland attempted to transmit the disease by inoculation, but without success.

Since we reported the transmission of leukemia in Strain C58, Korteweg (15) has recorded the transmission of "leukosarcomatosis" in mice. Korteweg's results are very similar to our own and, as will be seen, the "leukosarcomatoses" and "leukemias" which occurred in the course of our experiments are interpreted as varieties of the same disease. Korteweg found leukosarcomatosis to behave, on transfer, like a true tumor. His material differs from ours, however, in that the mice used for inoculation were not from a single strain, and were thus very different genetically. Furthermore, all of his material came originally from the tumor of a single mouse.

### *Spontaneous Leukemia in Strain C58*

The cases of lymphatic leukemia and related conditions which have been observed in Strain C58 are similar in their anatomical and microscopical features to those reported by other observers. The varieties observed by Simonds (13) in the Slye stock have also been found in Strain C58. It will be necessary, however, to note certain general features of the disease as it has occurred in this strain.

*Incidence.*—A large proportion of mice of both sexes have had leukemia. Of 155 mice that died since May, 1928, 137 had leukemia. This, however, is not a final result as many mice are still alive. There are wide variations in the age of incidence and some animals have died at relatively early ages. The figures should not, therefore, be read in terms of percentage. An account of the history of leukemia in Strain C58 will be published in a subsequent report.

*Age Incidence.*—In 137 cases of spontaneous leukemia that died since May, 1928, the age at death is given in Table I. The youngest animal in this series was 186 days old. 104 or 76 per cent of the cases occurred between the 8th and 12th months; 18 or 13 per cent were less than 8 months old, and 15 or 11 per cent were over 1 year.

During the same interval, 18 other mice died and 4 were killed. Of these, 6 were diagnosed as "doubtful" (usually because of extreme post-mortem decomposition), and 16 as "not leukemia."

*Occurrence of Other Diseases.*—In the early history of the strain, many animals were discarded before the age at which neoplasms are commonly found. Since March, 1928, at which time discarding of animals was discontinued and necropsies regularly performed, a few neoplasms have been found other than those of the leukemic group under discussion. These include one case of epithelioma of the skin,

TABLE I

	Age in months																	
	6	7	8	9	10	11	12	13	14	15	16	17	18	19				
No. of animals.....	5	13	19	35	24	26	5	2	2	2	2	1	0	1				

one of osteogenic sarcoma and two of carcinoma of the lung, all of which were associated with leukemia. There were also one case of chondro-osteosarcoma and one salivary gland tumor. Of other diseases, none has been constant. External parasites have occasionally appeared in the colony, and a few cestodes have been found.

*Diagnosis.*—The final diagnosis has, in each case, been made by gross and microscopic examination of the organs at autopsy. In typical cases the lesions are sufficiently characteristic to enable accurate diagnoses to be made by gross findings alone, although sections have been regularly examined. In only two cases were diagnoses of leukemia made at autopsy changed after examinations of the sections, but a few unsuspected cases of leukemia in early stages were found microscopically.

It is convenient to recognize the disease during life, and this we have found possible in a very large number of cases. In general, en-

largement of the spleen is the first symptom noted, and can be recognized by palpation of its lower free end and usually distinct edges. The number of cases detected in early stages has been roughly proportional to the regularity of periodic examinations of spleens in the colony. Although splenomegaly is also found in conditions other than leukemia, we have encountered but few such instances in these mice. There is also emaciation, causing the pelvic bones to become prominent, the nose sharp, and the vertebrae of the tail distinct. The abdomen becomes distended and feels firm and heavy in spite of the emaciation. The animal is less active and its movements slow. The presence of large intrathoracic tumors such as occur in the "leukosarcomatoses" has frequently been diagnosed by the occurrence of labored

TABLE II  
*Leucocyte Counts on 250 Normal Mice of Strain C58*

White cell count	No. of counts
5-10,000 per cmm.	81
10-15,000 " "	110
15-20,000 " "	64
20-25,000 " "	27
25-30,000 " "	5
30-35,000 " "	2
35-40,000 " "	1

breathing. Blood examinations often give valuable confirmatory evidence of the presence of leukemia, but the absence of a typical blood picture does not necessarily indicate the absence of leukemic changes in the tissues. The data in Table II of leucocyte counts in mice of Strain C58, compiled from 290 counts on 250 normal, uninoculated mice between the ages of 1 and 4 months may be used for comparison with the counts on cases of spontaneous and transmitted leukemia.

All of these mice were subsequently used for inoculation. From the table it will be seen that in only 3 of the 290 examinations were there more than 30,000 white blood cells per cubic millimeter. Blood smears were not examined in every instance, but neither in those which were examined nor in the counting chamber when the leucocytes were counted were abnormal cells observed. In addition, smears of the

blood of 175 mice less than 6 months of age, which were made in the spring of 1928, failed to show abnormal cells resembling those in the typical leukemic cases. Many of these animals later developed leukemia spontaneously.

In 131 cases of spontaneous leukemia, the distribution of the leucocyte count is given in Table III, the highest count for each mouse being used.

Of these, 6 were over 300,000 and 2 over 600,000. Thus in 22.9 per cent of these cases, the leucocyte count was never found to be above 30,000 per cubic millimeter, a figure occasionally reached in normal mice. In several cases with counts below 30,000, abnormal cells were seen in smears or in the counting chamber.

Of the 100 mice that at some time had leucocyte counts of 30,000

TABLE III

*Leucocyte Counts in Spontaneous Leukemia*

White cell count	No. of animals
under 30,000 per cmm.	31
30-50,000 " "	35
50-100,000 " "	35
over 100,000 " "	30

or more, 33 subsequently had diminution of the leucocytes, which in 18 cases were then less than 30,000. A few of these changes are, for example: from 66,000 to 11,000; 75,000 to 25,000; 354,000 to 31,900; 184,000 to 60,000; 229,000 to 3800 with subsequent rise to 46,000.

The abnormal cells which appear in the blood in typical cases of leukemia are larger than the normal mouse lymphocyte. The nuclei are large, usually round, and leptochromatic. When stained by one of the Romanowsky methods a nuclear network of chromatin is demonstrated, which is somewhat less dense than in the normal lymphocytes, though in both of these types of cells the structure is slightly more coarse than in the corresponding human leucocytes. Several nucleoli may be present, but are not constant. The cytoplasm is basophilic, frequently with small clear areas near the nucleus, or with small vacuoles. Specific granules are not present, but azure

granules are occasionally found. Mitoses have been observed. Many cells are intermediate in type between these immature forms and the normal lymphocytes.

*Course.*—No instance of recovery from spontaneous leukemia has been observed in this strain. The disease is progressive, but variations have been observed in the size of enlarged lymph nodes. There is increasing enlargement of the spleen, weakness, emaciation, and frequently marked anemia. In several cases the course was protracted, lasting in one instance 10 months after enlargement of the spleen was first noticed.

*Varieties of Manifestation, and Terminology.*—It is known that both myelogenous and lymphatic leukemia occur in mice, as well as related lymphoid hyperplastic and neoplastic conditions, which offer difficulties in classification. Some of the latter correspond to the "pseudo-leukemias," "leukosarcomatoses" and related conditions as observed in man. Hodgkin's disease appears to be an exception, for but five cases have been reported in mice (13, 16).

In Strain C58 the cases of leukemia which have been examined since the spring of 1928 have been only of the lymphatic type. Among these are mice showing the typical lesions of lymphatic leukemia including a characteristic blood picture, and also, in smaller numbers, those which would correspond to cases of "leukosarcomatosis" or "pseudoleukemia," as described by Simonds (13). In all cases, however, the type of cell comprising the infiltrations is of lymphoid nature. The similarity of these infiltrations, the variations in the blood picture and the occurrence of cases intermediate in type between leukemia and leukosarcomatosis or lymphosarcoma, give one the impression that these different anatomical varieties are but variations in the distribution of lesions rather than differences of fundamental nature.

The distinction between "leukemia" and "pseudoleukemia," the latter term referring to Cohnheim's designation of that condition in which the gross and microscopic lesions are characteristic of leukemia, but in which the blood picture does not show leukemic changes, should not, in our opinion, be made in mice of this strain. Repeated blood examinations in mice with palpably enlarged spleens have shown that:

- (1). Splenic enlargement frequently precedes the appearance of a leukemic blood picture;

(2) A leukemic blood picture usually occurs at some time during the course of the disease;

(3) The blood changes may be a very late manifestation;

(4) A leukemic blood picture is not always permanent, but may approach or reach normal without recovery or corresponding clinical changes.

In view of these variations in the blood picture, it is evident that the classification of a particular case as "leukemia" or "pseudoleukemia" depends on the stage at which death occurred, and that the blood picture is an inconstant symptom rather than an indication of fundamental differences. Likewise there are cases with lesions the distribution of which is intermediate between the diffuse changes of leukemia and the local growths of "leukosarcomatosis" or "lymphosarcoma." We therefore prefer to classify in one group all of those conditions which we believe to be fundamentally of the same nature. For this group the term "leukemia" is selected, not because it is literally descriptive, but because it is widely used and generally understood. "Pseudoleukemia," "leukosarcomatosis" and similar terms represent, as far as these mice are concerned, unimportant though interesting variations in the extent or distribution of the lesions.

### *Experimental Transmission of Leukemia in Strain C58*

The first of our experiments on the transmissibility of leukemia in this strain was performed as follows:

A female mouse (50420) not previously used in any experiment was first observed to have a large spleen at the age of  $7\frac{1}{2}$  months. At 10 months the spleen was larger, and the circulating white blood cells numbered  $69\frac{1}{2}$ ,000 per cubic millimeter, of which the great majority were lymphoid cells of immature type. The mouse was killed, the spleen removed aseptically, and a portion of it minced in sterile saline. Examination of the organs at autopsy showed enlargement of the spleen and liver and of the cervical, axillary, inguinal and mesenteric lymph nodes. The diagnosis of lymphatic leukemia was later confirmed microscopically.

The saline emulsion of spleen was immediately inoculated intraperitoneally into 8 mice of Strain C58 which were 4 weeks of age.

Following the inoculation, the leucocytes were counted at intervals and were found in each case to increase in number, reaching figures higher than are found in many cases of leukemia. The highest count found in this experiment was 262,000 about 3 weeks after inoculation. The increase in the number of white cells



appeared to be due to the presence in the blood stream of immature lymphocytes comparable to those of the spontaneous case.

Two of the 8 mice were killed at 17 and 21 days respectively after inoculation, while the rest were allowed to live until death occurred spontaneously in 32 to 35 days after inoculation. The gross appearance of Mouse 53983 shows the type of lesion found in this particular experiment, though it is not characteristic of all:

The spleen measured  $1.9 \times 0.5 \times 0.3$  cm. It was of normal color, the follicles were distinct but not larger than normal.

At the site of inoculation in the anterior abdominal wall was a tumor about  $1 \times 0.8$  cm. in diameter. Near it and on the opposite side of the abdomen, were smaller nodules in the abdominal wall. The cervical nodes were enlarged, slightly greater on the right side. Axillary and inguinal nodes were but slightly enlarged. In the mesentery were many nodules, each less than 0.2 cm. in diameter. A mass of lymphoid tissue was behind the pyloric portion of the stomach, between it and the liver. There was also infiltration of the perirenal fat, forming tumor-like masses on either side of the vertebral column, mesial to the kidneys.

The liver, lungs, kidneys, thymus and mediastinal lymph nodes were normal.

Microscopic examination showed the lesions to be composed of lymphoid cells of abnormal type, with many mitoses. Smears of the tissues show that these are the same type of cell described above in the blood of leukemic mice.

It is significant that in this and subsequent experiments, the lesions of leukemia were obtained in mice dying of the disease at an earlier age than that at which any mouse has been observed to have leukemia spontaneously, either in this strain or in any other recorded in the literature.

From one of the animals in this experiment, the spleen was removed aseptically, a saline emulsion prepared, and other mice of Strain C58\* inoculated. In this manner, by the inoculation of tissue emulsions\*\* the disease has been repeatedly transferred, and is now in its 30th passage. The experimental leukemia of this series, originating from Mouse 50420 is designated as "Line A," the first transfer as A1, the second as A2, etc.

\* We have not been successful in transmitting the disease to mice of other strains.

\*\* Our usual procedure has been to inoculate intraperitoneally with emulsion of spleen or lymph node. Positive results have also been obtained after the inoculation of ascitic fluid, which in some experiments has been abundant, with heart-blood and with liver. Subcutaneous inoculation is also successful, but the distribution of the lesions is somewhat different.

The experiment was repeated, using tissues of other mice with spontaneous leukemia, and the experimental leukemias thus obtained are designated as "Line B," "Line C," etc. In all, 10 spontaneous cases have been used as donors, with successful transmission in each. Some of these lines have been allowed to die out. Four lines are now used for continued transmission and the current experiment in each is A30, E10, H10 and I9.

The lesions found in Experiment A1 are fundamentally the same as those of subsequent experiments, but variations have been observed in the distribution of the lesions, the degree to which individual organs are involved, and the occurrence of leukemic blood pictures. These variations are comparable to similar differences in distribution of the lesions observed in the spontaneous cases. In each of the experimental lines now being studied, minor differences in the distribution of the lesions, the type of reaction and the interval between inoculation and death are observed which, though not perfectly constant, are sufficiently characteristic to indicate the presence of differences in the inocula. Thus, after intraperitoneal inoculation, Line A is now characterized by the nearly constant presence of ascites; Line E by the relatively frequent occurrence of chest tumors with fluid in the pleural cavity, but infrequent ascites; Line H by somewhat higher leucocyte counts and marked infiltrations in the liver, without chest tumors and only rarely with ascites; and Line I by marked infiltrations in the kidneys and liver, with high leucocyte counts, but without chest tumors or ascites.

In the later transfers of each experimental line there has been a decrease in the average interval between inoculation and death, and, at the same time, less variation among animals inoculated with the same material. This enables us to obtain, at present, fairly uniform results after inoculation, with each experimental line presenting its individual characteristics. An abstract of these results is appended.

Microscopically the lesions found in different experiments are similar, in that they all consist of the presence of numerous large cells of lymphoid type with frequent mitoses. These cells appear in the blood stream and infiltrate distant organs or tissues. In some experiments the cells have infiltrated the liver, spleen, kidneys and pancreas, producing the same picture as in spontaneous leukemia. In

Experiment	No. of mice	Positive results	Negative results	Average period of survival (days)*
<i>Line A</i>				
A1	8	8	0	33.5 (6)
A2	4	1	3	(a)
A3	25	20	5	28.19 (16)
A4a (b)	8	8	0	19.3 (6)
A4b	8	8	0	21.6 (7)
A4c	6	5	1	30.25 (4)
A5a (c)	6	6	0	12.6 (5)
A5b (c)	3	3	0	23.0 (2)
A6a (d)	4	4	0	16.25 (4)
A6b (e)	4	4	0	16.7 (3)
A6c (e)	4	3	1	22.0 (2)
A7a (f)	4	4	0	15.5 (4)
A7b (g)	4	4	0	20.3 (3)
A8	4	4	0	13.7 (3)
A9	4	2	2	(h)
A10	4	4 (i)	0	29.5 (2)
A11	4	2	2 (j)	20.0 (1)
A12	4	4	0	10.5 (2)
A13a	4	4	0	9.0 (3)
A13b	4	4	0	10.0 (4)
A14 (k)	8	8	0	10.8 (7)
A15	8	8	0	12.0 (7)
A16	4	4	0	15.7 (3)
A17	4	4	0	10.0 (3)
A18	8	8	0	9.4 (7)

\* The figures in parentheses indicate the number of positive cases from which the average is calculated. Animals that were killed are not included.

(a) The one positive case was killed 46 days after inoculation. One of the negatives died at 4 days.

(b) Three mice of A3 were killed and used as donors, hence the subdivisions of A4. Similarly for A5, A6, A7 and A13.

(c) Donor from A4a.

(d) Donor from A5a.

(e) Donor from A5b.

(f) Donor from A6b.

(g) Donor from A6c.

(h) One killed at 15 days was positive. One died at 141 days (age 206 days), may be a spontaneous case.

(i) One died at 147 days (age 214 days) may be a spontaneous case. Not counted in average duration.

(j) One died 5 days after inoculation with negative result.

Experiment	No. of mice	Positive results	Negative results	Average period of survival (days)
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*Line A—Concluded*

A19	12	10	2	13.4 (9)
A20	12	12	0	10.5 (11)
A21	12	12	0	10.5 (11)
A22	12	12	0	13.0 (11)
A23	8	8	0	13.0 (7)
A24	5	5	0	7.25 (4)
A25	8	7	1 (l)	11.6 (5)
A26	8	6	2	10.75 (5)
A27	8	8	0	13.7 (7)
A28	6	6	0	20.0 (4)
A29	8	8	0	14.1 (6)
A30	4	4	0	9.0 (2)

*Line B*

B1	12	5	7 (m)	54.5 (4)
B2	8	8	0 (n)	12.7 (6)
B3	8	2	6 (o)	17.0 (1)
B4	5	5 (p)	0	9.0 (3)

Discontinued

*Line C*

C1	7	6	1	58.1 (6)
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Discontinued

*Line D*

D1	12	3	9	28.0 (2)
D2	5	4	1	17.3 (3)
D3	6	6	0	18.3 (6)

Discontinued

(l) The negative died three days after inoculation.

(m) Four negatives died in less than 4 days.

(n) In one case diagnosis is not certain. Nodes and spleen were enlarged, but animal was decomposed and partly eaten by other mice.

(o) The 6 negatives died 1 day after inoculation.

(p) One case doubtful because of post mortem decomposition.

Experiment	No. of mice	Positive results	Negative results	Average period of survival (days)
<i>Line E</i>				
E1	9	8	1 (q)	66.8 (6)
E2	8	7	1 (r)	57.3 (6)
E3a	25	9	16	36.3 (8)
E3b	6	6	0	50.7 (6)
E4 (s)	4	4	0	51.3 (3)
E5	4	4	0	32.3 (3)
E6	4	4	0	25.0 (3)
E7	8	8	0	22.0 (7)
E8	8	8	0	26.8 (7)
E9	6	6	0	21.6 (5)
E10	8	8	0	19.5 (7)
<i>Line F</i>				
F1	5	4	1	15.3 (3)
F2	3	3	0	21.0 (3)
Discontinued				
<i>Line G</i>				
G1	8	8	0	48.25 (8)
Discontinued				
<i>Line H</i>				
H1	2	1	1 (t)	35.0 (1)
H2	6	6	0	30.5 (4)
H3	4	3	1 (u)	20.5 (2)
H4	7	7	0	16.1 (6)
H5	4	4	0	17.7 (3)
H6	4	4	0	18.7 (3)
H7	8	8	0	15.0 (7)
H8	8	8	0	14.0 (7)
H9	6	6	0	17.5 (5)
H10	6	6	0	18.6 (5)

(q) The negative case died 36 days after inoculation. Others became ill not less than 49 days. Two killed at 109 and 121 days were positive.

(r) Negative case died 1 day after inoculation.

(s) Donor from E3a.

(t) The negative case had a large spleen but was too much decomposed to diagnose microscopically.

(u) The negative case died 2 days after inoculation.

Experiment	No. of mice	Positive results	Negative results	Average period of survival (days)
<i>Line I</i>				
I1	4	4	0	55.0 (2)
I2a	4	1	3	(v)
I2b	6	6	0	29.0 (6)
I3	4	4	0	36.0 (3)
I4	4	4	0	20.0 (3)
I5	6	6	0	29.6 (5)
I6	8	8	0	25.7 (7)
I7	6	6	0	19.8 (5)
I8	6	6	0	13.4 (5)
I9	6	6	0	10.75 (5)

*Line J*

(Inoculated at Cold Spring Harbor)

J1	4	3	1	22.0 (3)
Discontinued				

(v) The one positive result was in an animal killed 14 days after inoculation and used as donor for I3. Emulsion kept on ice 48 hours before use. Two animals died 4 days after inoculation.

other cases the infiltration of these organs may be minimal or absent, yet the mesentery and omentum may have numerous small nodules, the fat tissue of the abdomen may be infiltrated and ascites may be present. These lesions will be reported in detail in a separate paper.

The appended protocols include only those mice of Strain C58 which were inoculated with emulsions of tissues not subjected to any preliminary treatment other than mincing in saline, with the exception of Experiment I3, the material for which was kept in a refrigerator for 48 hours before use. In calculating the average period of survival, only those mice that died of transmitted leukemia are included. The figure in parenthesis after each average indicates the number of mice from which the average is calculated. Except where noted, the inoculations were made in New York.

In addition to these C58 animals, there were inoculated 107 mice from the Storrs-Little strain, 19 of Dilute Brown, 16 Bagg albinos, and 3 of Strain 85, all with negative results.

A series of experiments performed in the laboratory at Cold Spring Harbor has given results similar to the above obtained in New York. A mouse from Experiment A17 and one from A25 were sent to Cold Spring Harbor and used as the donors in subsequent experiments, and the series of transmissions obtained therefrom correspond to those in this line reported in the protocols.

Of 78 mice thus inoculated, 73 developed leukemia, and 5 died in less than 4 days after inoculation.

#### SUMMARY

Lymphatic leukemia has occurred with great frequency in a particular strain of mice which have been inbred by brother-sister matings since 1921. In addition to typical cases of leukemia are others which, because of the absence of leukemic changes in the blood, correspond to "pseudoleukemia" and others which, by the presence of unusually great enlargement of certain lymph node groups resemble the "leukosarcomatoses" as observed in man.

Examinations of the blood of leukemic mice have shown that leukemic blood pictures are not necessarily early in their appearance, nor are they constant. The blood picture may not, therefore, be used as a criterion for the separation of the two diseases (leukemia and pseudoleukemia) but merely indicates different phases of the same condition. Likewise, cases with lesions intermediate between the local growths of "leukosarcomatosis" and the more general lymphatic enlargements of leukemia suggest that these conditions differ only in the distribution of lesions but not in their nature.

Lymphatic leukemia occurring spontaneously in this strain may be transmitted to other mice of the same strain, and carried, apparently, for an unlimited number of transfers in animals at an earlier age than that at which leukemia occurs spontaneously. In each of 10 such experiments transmissions were obtained. The lesions produced by inoculation correspond to those of spontaneous cases, in that they consist of growths of abnormal lymphoid cells which infiltrate tissues and organs and often appear in the circulating blood. Only minor differences have occurred, some of which are characteristic of certain experimental lines. After repeated transfers, the disease tends to run a more acute course.

Among the cases in which transmissions occurred, are some without leukemic changes in the blood, and many with local growths at the site of inoculation or in certain node groups. The differences in the blood pictures and distribution of lesions (which latter may be influenced to some extent by the method of inoculation) correspond to similar differences which are sometimes observed in the spontaneous cases.

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## OBSERVATIONS ON THE PHAGOCYTOSIS OF THE PNEUMOCOCCUS BY HUMAN WHOLE BLOOD

### I. THE NORMAL PHAGOCYTIC TITRE, AND THE ANTI-PHAGOCYTIC EFFECT OF THE SPECIFIC SOLUBLE SUBSTANCE

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It is generally agreed that the human body depends largely on the phagocytic mechanism in repelling an invasion by the pneumococcus. The conditions, however, under which this organism gains an entrance and causes pneumonia are still little understood. We say that the patient's resistance was lowered at the time of infection, but this phrase "lowered resistance" helps us very little towards an understanding of what it is in the normal resistance that breaks down. We are uncertain whether the infection is endogenous or exogenous or, as is more probable, is either; whether the mechanism of resistance depends on the phagocytic action of the blood alone, or, as is more probable, of a combination of blood and fixed cells; whether resistance is lowered generally against all the strains of pneumococcus, or whether it is naturally low against the type which infects; whether the resistance of the whole body is lowered, or is only lowered locally from one cause or another, at the point where the pneumococcus invades the tissues. The approach to the solution of many of these problems is obviously difficult in experimental animals, and still more difficult in the human subject. There is, however, one of these problems which lends itself to experimental investigation, *viz.*, the phagocytic titre of human whole blood against each of the three well-known types of the pneumococcus. It is not contended for a moment that the phagocytic titre of the blood is a complete expression of the resistance of the individual against the pneumococcus, but it is reasonable to assume that it is a factor in that

resistance, and—short of actually infecting the individual with living organisms—the only way available at present of obtaining any quantitative expression of general resistance, however imperfect it may be. The “phagocytic titre” of a blood is expressed in the number of organisms a certain amount of the blood will phagocyte and kill, the organisms being incubated in the blood under conditions suitable for phagocytosis. No attempt has been made to standardize quantities, times or conditions, but the experiments were all uniform in these respects so that the results obtained can be compared with one another.

The method employed in determining the phagocytic titre of the blood is a modification of one described by Todd (1), and used by him for another purpose, *viz.*, the estimation of the comparative virulence of certain strains of haemolytic streptococci.

In this method, a constant amount of whole blood is placed in a series of tubes, and to each of the tubes is added a decreasing number of organisms, so that—for example—the first tube is inoculated with 500,000 organisms, the second tube with 50,000 organisms, the third tube with 5000 organisms, and so on. The tubes are sealed and placed in a rotating box in the incubator. After some hours' incubation, the tubes are opened and the contents plated out. The plates are incubated and read next day. It is evident that whether the contents of the tubes are sterile or not—as shown by the plates—depends on three main factors, *viz.*, the number of organisms, the virulence of the organisms, and the phagocytic power of the whole blood. As the number of organisms can easily be controlled, the method can be used to determine either the virulence of the organisms, by always using the same blood, or the phagocytic power of the blood by always using the same organisms, if their virulence can be maintained at a constant level. The maximum number of organisms killed is in the one case the direct measure of the phagocytic power of the blood, and in the other case the indirect measure of the virulence of the organism. In other words, the more organisms that are killed, the more actively phagocytic is the blood in the former case, and the less virulent is the organism in the latter case.

The technique that has been used in these experiments for determining the relative phagocytic titres of certain human bloods will now be described. It follows fairly closely that used by Todd in his experiments (1).

The special apparatus necessary is, firstly, a supply of pyrex tubes, about 10 cm. long, 7 mm. inside diameter. These can be made very easily from pyrex tubing, using an oxygen flame. Care should be taken that the bottom of the tube is

rounded off, and not drawn out into a point. The open end of the tube is plugged with cotton wool, and the tubes sterilized in the hot air oven. Just before starting the experiment, the cotton wool plugs are removed, and the open end of the tubes covered with sterile glass caps, as these are more convenient to remove and replace than the wool plugs. The caps can be made from tubing whose inside diameter is about 10 mm.

Secondly, a rotating mechanism, so that the tubes can be rotated about their long axis, while they are being incubated. Rotation is essential, because if the tubes are simply allowed to stand in the incubator, the leucocytes sink to the bottom of the tube and the organisms grow in the cell-free upper layers of the blood. A simple form of rotating machine is made by coupling a 1/20 horse power motor (2000 r.p.m.) to a speed-reducing gear (48:1) with a loose leather belt, and then with a similar belt coupling the speed reducing gear to a shaft carried in two bearings placed about 30 cm. apart. On opposite sides of this shaft, between the bearings, are firmly bolted two wooden boxes, 23 cm. long, 15 cm. wide, and 5 cm. deep. Each box is provided with a hinged lid. The motor, speed-reducing gear, and the supports carrying the bearings for the shaft, are mounted on a board. The supports for the shaft bearings should be tall enough to permit the boxes to swing clear of the board. The secondary shaft of the speed-reducing gear rotates about eighteen times per minute and a further reduction is necessary. By using pulley-wheels of suitable size on the secondary shaft and on the shaft carrying the boxes, the revolutions of the latter shaft are reduced to about six per minute, which is a convenient rate. The apparatus is placed in the incubator and the motor connected up. When the tubes are filled and sealed, they are placed in the boxes with their long axis across the long axis of the shaft, packed in firmly with wool, the lid closed down, and the motor started. The special receptacle for each tube described by Todd was not thought necessary.

Although not essential, a special Dreyer pipette for use with the tubes is a convenience. This pipette is made of tubing whose outside diameter is 5 mm. The body of the pipette is about 15 cm. long. One end is lipped for use with a rubber nipple, and the other end is drawn out into a thick-walled capillary, 25 mm. long. This pipette is graduated to deliver 0.5 cc. from one mark on the body of the pipette to the tip of the capillary, and 1.0 cc. from a second mark on the body of the pipette to the tip of the capillary.

The individual whose blood is to be tested is bled from the arm vein, and the blood placed in a sterile flask, containing glass beads. The flask is agitated continuously, until defibrination is complete. For reasons which will be considered later, it is important that the blood should be defibrinated thoroughly. 0.5 cc. of blood is then placed in the bottom of each tube, filling as many tubes as are necessary for the experiment. Care should be taken not to get any blood on the upper part of the tube, as it would be charred in the subsequent sealing of the tubes. If the special pipette described above is used to fill the tubes, it is first cleaned and sterilized by connecting it with a suction apparatus, drawing water,

alcohol and ether, successively, through the pipette, and then flaming it. A rubber nipple is fitted to the pipette, and with a little practice, the tubes can be quickly and cleanly filled with the required amount of blood. If an ordinary sterile graduated pipette is used, it should be quite thin, so that the tip can be placed in the bottom of the tube, before allowing the blood to run

The organisms to be used, in these experiments pneumococcus Types I, II and III, are incubated for 18 hours in hormone broth, to which a drop of rabbit blood has been added. Their virulence is of course tested on mice from time to time to insure that they are cultures of maximum virulence. As a further check, the cultures are plated out in each experiment and the colonies examined for smoothness. Just prior to adding the organisms to the tubes containing the blood, the culture is diluted successively in hormone broth, the dilutions used being 1/10, 1/100, 1/1000, 1/10,000, 1/100,000 and 1/1,000,000 of the original culture. With the special Dreyer pipette, one drop of the 1/10 dilution of culture is added to the first tube of blood, one drop of the 1/100 dilution to the next tube, and so on. The actual amount of fluid in one drop of the culture dilution can be readily calculated for a particular pipette by ascertaining the number of drops which will exactly fill a 10 cc. graduate. The drop is used instead of the delivery of a certain amount, say 0.1 cc., from a graduated pipette, because, in the first place, the drop is a more accurate measurement, and, in the second place, it was thought desirable to disturb the concentration of the various constituents of the blood as little as possible by adding the smallest possible measured amount of culture. Finally, a drop (of course from the same pipette) of the 1/100,000 dilution of the culture is allowed to fall on a blood plate. The drop is spread out, the plate incubated, and the colonies counted the next day. In this way, one can estimate approximately the number of diplococci added to each tube.

In an actual experiment to estimate the phagocytic power of a particular blood against the three types of pneumococcus, there would be in all eighteen tubes, six tubes inoculated with the Type I culture in its various dilutions from 1/10 to 1/1,000,000, six tubes with Type II, six tubes with Type III. After the tubes have been inoculated, the glass caps are removed, and the open ends sealed in an oxygen flame, care again being taken to round off the end of the tube. The tubes are then packed into the boxes of the rotating apparatus in the incubator, and the motor started. The next day, the tubes are taken out of the apparatus, the upper ends flamed, nicked with a file, and the tube broken open. One loopful from each tube is then streaked out on a blood agar plate. Usually, six tubes are streaked on one plate. The plates are incubated, and the amount of growth estimated on the following day. In the tables in the text, the following signs are employed to indicate the amount of growth: Maximum growth = + + + +; Near maximum growth = + + +; Moderate growth = + +; Slight growth = +; Sterility = O. In the tubes with maximum growth, the oxygen in the tubes is used up, and the haemoglobin reduced, so that the blood appears very dark red to black in color. It is unwise, however, to rely on the color, as a tube containing dark blood may pos-

sibly be contaminated, and a tube containing red blood may contain some living organisms.

Ten normal adult males were tested in this way, with the results given in Table I.

A repetition of the experiment produced similar results. In addition, it was found that Blood A could not kill ten times the maximum

TABLE I

Type of organism	Approximate number of diplococci inoculated	Blood A	Blood B	Blood C	Blood D	Blood E	Blood F	Blood G	Blood H	Blood I	Blood J
I	300,000	0	+++	++++	0	++++	++++	++++	++++	++++	++++
	30,000	0	0	++++	0	+++	++++	++++	+++	++++	0
	3,000	0	0	++	0	0	++++	++++	0	+	0
	300	0	0	+	0	0	++++	++++	0	0	0
	30	0	0	0	0	0	++++	0	0	0	0
	3	0	0	0	0	0	++++	0	0	0	0
II	600,000	0	+	++++	++	++++	+++	++	+	++++	++++
	60,000	0	0	++	0	+++	0	0	0	++++	++++
	6,000	0	0	0	0	0	0	0	0	0	0
	600	0	0	0	0	0	0	0	0	0	0
	60	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0	0
III	800,000	++++	+++	++++	++++	++	++++	++++	++++	++++	++++
	80,000	++	0	+++	++++	0	++	0	++++	+	++
	8,000	0	0	0	0	0	0	0	++++	0	0
	800	0	0	0	0	0	0	0	++++	0	0
	80	0	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	0	0	0	0	0	0

number of Type I and Type II organisms shown in this table, nor could Blood D kill ten times the maximum number of Type I organisms shown in the table. Table I, then, portrays very fairly the maximum number of virulent organisms these bloods were able to destroy.

Striking differences are seen in the phagocytic titre.

Blood A and Blood D can kill at least 300,000 Type I organisms, while Blood F cannot even kill 3 diplococci of this type. Weakness against one type is not

associated with weakness against the other types, as is shown by Blood F and Blood G. Subsequent tests which were carried out from time to time showed that the titre apparently remained constant, except in the case of Blood C. This individual was frequently bled some 40 to 50 cc. over a period of 2 or 3 months. At the end of this period, his phagocytic titre had definitely fallen against all three types.

As was to be expected, the cells in these specimens of blood were equally effective, whether the titre was high or low.

Thus, in the case of Blood A and Blood G, the phagocytic titre of A's serum and G's washed corpuscles was equal to the phagocytic titre of A's whole blood, and G's serum and A's washed corpuscles were only able to phagocyte as many organisms as G's whole blood.

Having obtained in this way an estimate of the phagocytic titre of normal human blood, and the range of variation that can occur in healthy subjects, attention was then turned to individuals infected with pneumococci. Blood was withdrawn from cases of pneumonia in the early stages before antiserum had been given, and tested by this technique. Suffice it to say that the phagocytic titre of such blood was well within the normal variation, and was not even depressed against the type of pneumococcus which was responsible for the pneumonia.

For example, a child, suffering from a Type IV infection, with irregular temperature and intermittent bacteraemia, had a high titre against her own organism. As post-operative pneumonias are not uncommon after operations in the upper abdominal area, an examination of the phagocytic titre was carried out on the day before and the day after operation, in order to find out whether the shock of operation lowered the titre. No change in titre was found, and in one of these cases, a mild pneumonia developed.

A few experiments were done with animal's blood. For reasons which are not wholly understood at present, inconstant and unsatisfactory results were obtained with rabbit whole blood, both normal and immune. The whole blood of a Type I immunized horse was able to kill 500,000 Type I diplococci, but was unable to kill ten times that number. Thus, it is on a par with the blood of A and D, normal individuals. Of course, it is realized that the serum of this horse's blood could have been diluted many times and still have been just as efficient in a phagocytic system, whereas the serum of the normal human blood

would not retain its efficiency, if much diluted. And therein lie the limitations of this simple test as outlined above—it does not give a measure of the opsonic content of the serum, if that content rises above a certain level, as it certainly does after immunization. In order to do this, a more complicated test is necessary—the immune serum must be diluted progressively and added to normal blood (see Paper II). There are at least two limiting factors in the simple test, when immune blood is used. One of these is the anti-phagocytic action of the precipitate formed when antiserum is mixed with undiluted culture fluid containing the specific carbohydrate (see Paper II), so that one is limited in the amount of culture one can add to the blood. The second limiting factor is the number of leucocytes available for phagocytosis, as is shown in the following experiment.

TABLE II

Approximate number of Type II diplococci inoculated	Blood mixture A, 5000 leucocytes	Blood mixture B, 3630 leucocytes	Blood mixture C, 2000 leucocytes	Blood mixture D, 1000 leucocytes
400,000	0	++++	++++	++++
40,000	0	0	++++	++++
4,000	0	0	++++	++++
400	0	0	++++	++++
40	0	0	++++	++++
4	0	0	0	++++

A quantity of normal human whole blood was centrifuged, the serum removed, and the cells thoroughly washed with normal saline solution. Mixtures were then made of the serum and the washed cells, so that the number of leucocytes diminished from the normal count to 1000 cells per cubic millimeter. These mixtures were then tested for the maximum number of organisms each would phagocytose, with the result seen in Table II.

From Table II it is seen that as the number of leucocytes diminish, so do the maximum number of organisms that are phagocytosed.

A very striking drop takes place when the number of leucocytes falls from 3600 to 2000. The reason for this critical change—the fall in leucocytes being out of all proportion to the fall in number of diplococci phagocytosed—is not known. Attention was drawn to it in the first place by a specimen of blood showing an unaccountable fall in phagocytic titre against all three types. The leucocyte count was only



1500 per cubic millimeter, and examination showed that it had partially clotted during defibrination with consequent entangling of a great number of leucocytes in the clot.

In the case of normal blood, there are also at least two limiting factors in the simple test. The first is the number of leucocytes available, and the second is the anti-phagocytic action of the specific carbohydrate in the culture fluid. An immune serum can easily neutralize a large amount of this substance, but a normal serum can only neutralize a very small amount. This antagonistic action of the specific carbohydrate on phagocytosis has been studied by Wadsworth

TABLE III

Type of organism	Approximate number of diplococci inoculated	Blood only	Blood + 1/100,000 concentration of Type I spec. sol. subst.	Blood + 1/100,000 concentration of Type III spec. sol. subst.
I	250,000	++	++++	++++
	25,000	0	++	0
	2,500	0	0	0
	250	0	0	0
	25	0	0	0
	2	0	0	0
III	300,000	++++	++++	++++
	30,000	0	0	++++
	3,000	0	0	++++
	300	0	0	++++
	30	0	0	++++
	3	0	0	0

and Sickles (2), who worked with pneumococcus filtrates, using the opsonic technique, and by Sia (3), who worked with the carbohydrate and used the more elaborate phagocytic technique developed by Robertson and Sia (4). In the present study, Sia's work was confirmed. It was found that the specific carbohydrate of Type I was anti-phagocytic when added to blood inoculated with Type I organisms, but had no such action when the blood was inoculated with organisms of the other types. In other words, its anti-phagocytic action is specific. It was also found that weight for weight, the Type III specific soluble substance was far more powerful in its anti-phago-

cytic action than the Type I carbohydrate. No Type II carbohydrate was available, but it is probable that its action is intermediate between that of Type I and that of Type III. Further, it can easily be demonstrated that, when grown in broth, the Type III organism produces much more precipitinogen, and the production is more rapid than in the case of Type I, and there is every reason to believe that the precipitinogen found in the culture fluid is either the specific soluble substance itself, or a substance closely akin to it. Some of these points are demonstrated in Table III.

The significance of these differences between the production and the actions of Type I and Type III specific soluble substances will be referred to again in Part II, which deals with the neutralization of these substances by their specific antisera. The specific soluble substance is considered here, because its presence in culture fluid limits the number of organisms that normal blood can phagocyte.

#### DISCUSSION

The phagocytic titre of whole blood against the three types of pneumococcus varies considerably in different individuals, and varies in each individual against each of the three types. There would, however, appear to be no grounds for believing that the phagocytic titre of normal human blood, as measured by this technique, is a very important factor in determining infection. It is obviously impossible to obtain the titre of an individual just before he comes down with pneumonia, but early in the disease, there is certainly no marked depression of the titre against the infecting organism. Moreover, the shock of operation has no influence on the titre, so that can not be a factor in post-operative pneumonia. If no lowering of resistance can be detected in the blood, it is unlikely that it is a general condition. This would, of course, point to a local breakdown in resistance as the important factor in pneumococcus infection. In this connection the work of Powers (5) should be considered. This author found that the incidence of post-operative pneumonias was much more frequent in operations in the upper abdomen than in other regions of the body, and that after these operations, the vital capacity of the lungs falls to a very low level. The correlation between the incidence of post-operative pneumonia and lowered vital capacity is clear, but it is not so clear

how the latter affects the tissues, so that the pneumococcus can gain an entrance. In view of the very considerable number of organisms that the average blood can phagocyte, tissue whose blood supply is partially or completely shut off might well be the starting point of the infection. Once the infection has started, the anti-phagocytic action of the specific carbohydrate would favor its local spread.

#### CONCLUSIONS

1. The phagocytic titre of whole human blood against the three types of pneumococcus was determined in a number of individuals. The titre varied over a considerable range in different subjects.

2. Contrary to expectation, the titre in early cases of untreated pneumonia was quite high against the infecting organism, pointing to a local rather than a general lowering of resistance in infection with this organism.

3. Sia's work was confirmed, that the specific carbohydrate has a specific anti-phagocytic action on the blood. This action is more marked in the case of Type III than in Type I.

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# OBSERVATIONS ON THE PHAGOCYTOSIS OF THE PNEUMOCOCCUS BY HUMAN WHOLE BLOOD

## II. THE NEUTRALIZATION OF THE ANTI-PHAGOCYTIC ACTION OF THE SPECIFIC SOLUBLE SUBSTANCE BY ANTISERUM

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The opsonic action of human serum from cases of pneumonia has been studied very thoroughly by Sia, Robertson and Woo (1), using their own technique. They found that the opsonic titre of the serum rose rapidly during and just after the crisis of the disease, and slowly decreased in the weeks that followed. This was so regular an occurrence that they were naturally inclined to believe that the crisis was the result of this rise. Other authors have noted that the crisis is contemporaneous with the appearance of mouse-protective substances in the serum. Indeed, in most of the work that has been done on the serum in pneumonia, attention has been focussed on these very striking changes that occur about the period of crisis, and which are almost certainly causally connected with that phenomenon.

The present study is not concerned with the direct phagocytic action of the blood of individuals suffering from pneumonia, for the blood in the early stages of pneumonia appears to act in this respect like the blood of healthy individuals (see Paper I), and as has been mentioned above, the blood at crisis has already been studied by Sia, Robertson and Woo. It is an attempt, however crude, to reproduce in the test tube the struggle that must go on in the lungs in serum-treated cases between the pneumococcus and its products on the one hand, and the blood and antiserum on the other. Admittedly, the reproduction is incomplete, because—owing to experimental limitations—one must leave out the tissue cells altogether, but at least it is possible to study the

interplay of the above factors on one another. If one studies under the microscope two opsonic preparations, one with normal human blood and virulent pneumococci, and the other with normal human blood and non-virulent pneumococci, a very different picture is seen. In the former case, the leucocytes are almost all empty, in the latter case, they are filled with pneumococci. If specific antiserum is added to the former preparation, the leucocytes now take up the organisms readily. We know that the virulent organisms are capsulated and contain the specific soluble substance, and that the non-virulent organisms are not capsulated and contain no specific soluble substance. We know, further, that the specific soluble substance has a specific anti-phagocytic effect—see Paper I. It would appear probable, then—as Dr. J. M. Alston suggested to the author—that the so-called “sensitizing” action of a specific antiserum is really a “neutralizing” action, the substance neutralized being the capsule composed largely of specific soluble substance. When this substance is neutralized, the capsulated, virulent pneumococcus behaves like a non-capsulated, non-virulent pneumococcus and is easily phagocyted. It was demonstrated many years ago by Dochez and Avery (2) that the specific soluble substance of the pneumococcus could be detected in the urine in cases of pneumonia during the course of the disease. Presumably this substance is set free in the lesion itself and it is reasonable to assume that if it can be detected in the urine, it must be present in still greater concentration in the lungs, though what the actual concentration there may be is not known. Granting this assumption, the struggle in the lungs in serum-treated cases would seem to narrow down to one between the antiserum on the one hand, and the specific soluble substance (both free and in the capsules of the living pneumococci) on the other, with the phagocytes as the arbiters of the outcome. It would be natural to suppose that if, after the free specific soluble substance had been neutralized by the antiserum, there was enough free antiserum remaining to neutralize the specific soluble substance in the capsules of the living pneumococci, these would be phagocyted, and the patient speedily recover; but that if the specific soluble substance remained in excess, there would be no free antiserum to “sensitize” the pneumococci, which would therefore not be phagocyted, and the patient’s life would be endangered. On this supposition, the problem

of serum therapy would resolve itself simply into giving enough anti-serum to neutralize the specific soluble substance; in other words, giving as much as possible, as often as possible, of a serum which is as strong as possible. And that is the usual practice in modern serum therapy in pneumonia.

The following experiments indicate, however, that very little anti-serum is necessary to neutralize comparatively strong concentrations of specific soluble substance, and that if that amount of antiserum is exceeded, phagocytosis is inhibited.

In Paper I, it was shown—using Todd's technique—that normal human whole blood was able to phagocyte very considerable numbers of virulent pneumococci, and that the specific carbohydrate had a specific antiphagocytic action on the blood, much more marked in the case of Type III than with Type I. An attempt was now made to study the neutralizing power of the specific antiserum on the specific carbohydrate, using phagocytosis of the type organism by whole blood as the indicator of whether the carbohydrate had been neutralized or not. The Type III system was first studied, and—to the author—quite surprising results were obtained.

A number of tubes were filled with 0.5 cc. of defibrinated human blood. Two drops of a 1/10,000 concentration of Type III specific soluble substance in normal saline were added to all the tubes (except the control series). The tubes were then divided into sets of six, and in each set, one drop of Type III horse antiserum of a certain dilution was added to each of the six tubes. The dilutions of the serum were made in normal saline solution and the following dilutions were commonly employed: (1) undiluted serum; (2) 1/4; (3) 1/16; (4) 1/64; (5) 1/256; (6) 1/1000; (7) 1/4000; (8) 1/16,000; (9) 1/64,000; (10) no serum. To these ten series was added a control series of six tubes, containing neither specific soluble substance nor serum, in order to show the normal phagocytic titre of the blood. To the first tube in each of these eleven series was then added one drop of a 1/10 dilution in broth of an 18-hour culture of Type III pneumococcus in rabbit blood hormone broth. To the second tube in each series a drop of a 1/100 dilution of the culture, and so on. The tubes were then sealed and incubated over night in the rotating apparatus. The next day the tubes were opened and a loopful of the contents streaked on blood agar plates. The plates were incubated for 24 hours, and the amount of growth from the streaks was roughly estimated. The result is set out in Table I. It shows the estimated concentration of both the specific soluble substance and the antiserum in the blood, and the estimated number of diplococci which were added to the tubes.

This experiment was repeated with the blood of another individual with a lower phagocytic titre, and also with a 1/750,000 concentration of specific soluble substance. There were slight variations from Table I, but there was the same general picture. Table I shows at a glance that it is not simply an excess of antiserum which determines the most efficient phagocytosis. For example, in Series I, it can easily be shown that the antiserum is in excess. If the blood is replaced by saline, and the precipitate formed by the specific soluble substance and the anti-

TABLE I

*Type III*

Number of diplococci inoculated	Series										
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
	Concentration of spec. sol. sub.										
	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	0
	Concentration of antiserum										
	1/20	1/80	1/320	1/1,280	1/5,000	1/20,000	1/80,000	1/320,000	1/1,128,000	0	0
300,000	++	++	++++	++++	+	+	++++	++++	++++	++++	++++
30,000	++	++	++++	++++	0	0	0	++++	++++	++++	0
3,000	+++	+++	+++	++	0	0	0	++++	++++	++++	0
300	+++	+++	+++	0	0	0	0	++++	++++	++++	0
30	+++	+	++	0	0	0	0	0	++++	++++	0
3	+	++	0	0	0	0	0	0	0	0	0

serum is centrifuged down, the addition of more specific soluble substance to the supernatant fluid results in another precipitate forming, showing there was an excess of antiserum in the original mixture. In the eighth and ninth series, where the specific soluble substance is in excess, the phagocytosis is again inefficient, as was to be expected, for the tenth series shows the powerful antiphagocytic action of specific soluble substance on the normal phagocytic power of the blood, shown in the eleventh series. In between the series where the antiserum is in

marked excess and the series where the specific soluble substance is in excess is a zone—series V, VI and VII—where phagocytosis is efficient. Here, in all probability, the antiserum is in slight excess, or there is a neutral mixture of specific soluble substance and antiserum.

A similar experiment was then carried out with Type I specific soluble substance and Type I antiserum, and no pro-zone of inefficient phagocytosis was found, so that it was thought at first that the phenomenon shown with Type III did not occur in the case of Type I.

TABLE II

*Type I*

Number of diplococci inoculated	Series										
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
	Concentration of spec. sol. sub.										
	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	1/75,000	0
	Concentration of antiserum										
	1/20	1/80	1/320	1/1,280	1/5,000	1/20,000	1/80,000	1/320,000	1/1,128,000	0	0
250,000	++	++	+++	0	0	0	+++	++++	++++	++++	++
25,000	+++	++	+++	0	0	0	0	+++	++	+++	0
2,500	++	++	0	0	0	0	0	0	0	0	0
250	+	+	0	0	0	0	0	0	0	0	0
25	+	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0

However, the Type I antiserum used on this occasion was a weak one, and later a very much stronger antiserum was obtained, containing about 2000 units. With this antiserum a Type I experiment was set up similar in every way to the Type III experiment. The antiserum used in all these experiments, whether Type I or Type III, was the natural serum not concentrated.

The result of the Type I experiment, using the strong antiserum, is shown in Table II. Here, with Type I, is seen the same phenomenon



that was demonstrated with Type III, but very much less marked, so that it is evidently common to all the strains of pneumococcus, varying only in degree. No Type II specific soluble substance was available, so that a Type II experiment could not be carried out, but from what we know of Type II, it is likely that it would give a result intermediate between Type I and Type III.

Since it might be argued that the addition to the tubes of specific soluble substance—a highly purified product of pneumococcus metabolism—is a somewhat artificial procedure, and that the unpurified product might have a different action, an experiment was carried out, using the undiluted filtrate of a 20 hour broth culture of Type I pneumococcus, instead of the specific soluble substance. The result was in every way similar to that shown in Table II.

An attempt has been made to find an explanation of why there is interference with phagocytosis in the series with considerable excess of antiserum. It is well known that in the precipitin reaction, if the antiserum is in sufficient excess, the precipitate formed is dissolved in the antiserum, the so-called “pro-zone” or inhibition zone. But the precipitin inhibition zone has only a very indirect bearing on the phenomenon described above, which might be called the phagocytic inhibition zone. If the blood in the various series is replaced with saline, one can observe the precipitates formed in each series. There is a precipitate visible in the first series, diminishing in amount as the serum is diluted in the later series. This in itself pointed to the precipitate being the interfering factor, so a simple experiment was set up.

Specific soluble substance and antiserum—both Type III—were mixed in suitable concentration so that a heavy precipitate formed. The precipitate was centrifuged down and the clear supernatant fluid pipetted off. The precipitate was washed three times in saline solution and then re-suspended in saline. Three series of tubes were set up with 0.5 cc. of whole blood in each tube. To the first series, one drop of saline was added; to the second series one drop of re-suspended washed precipitate, and to the third series one drop of the supernatant fluid. Each series was then inoculated with Type III culture in the usual way. The tubes were sealed, incubated in the rotating apparatus and streaked out on the following day. Table III shows the result.

This experiment brings out very clearly that it is the precipitate which interferes with phagocytosis, and that the clear supernatant

fluid of the antiserum-specific carbohydrate mixture reinforces the natural phagocytic power of the blood.

The next question of course that arises is: Where does the precipitate act? Does it interfere with the leucocytes themselves, or does it exert its inhibiting action in some way on the serum, perhaps adsorbing some substance from the serum which is necessary for phagocytosis? With regard to the latter possibility, it is not inconceivable that phagocytosis would be more effective in the presence than in the absence of complement, and it is known that complement is usually adsorbed by specific precipitates. Would the adsorption of complement by the specific precipitate explain, then, the inefficient phagocytosis? This possibility was investigated by incubating for 2 hours a tube containing the uninoculated mixture in Series I of Table I (con-

TABLE III

Number of diplococci inoculated	Blood + saline	Blood + washed precipitate	Blood + supernatant fluid
700,000	++++	++	+
70,000	+++	++	0
7,000	+	+++	0
700	0	+++	0
70	0	+++	0
7	0	+	0

sisting of blood, Type III antiserum and Type III specific carbohydrate) and also a tube containing the uninoculated Series XI of Table I (consisting of blood only). The serum was then pipetted off both tubes and tested for complementing power with sensitized red cells. It was found that the complementing power of the serum, where the precipitate was present, had fallen to half that of the control tube, but that there was still a large amount of complement remaining. That more complement was not adsorbed by the precipitate appears surprising, but this result is characteristic of the pneumococcus antigen-antibody complex, where horse serum is the source of the antibody, as Zinsser and Parker (3) have shown, using guinea pig complement.

That the precipitate does not exert its action on the serum can be shown in another way, by the following experiment:

An equal amount of human whole blood was placed into two centrifuge tubes. The tubes were centrifuged, and the serum pipetted off and kept separate. The corpuscles in each tube were washed three times in saline solution. The serum from Tube 1 was then replaced on the washed corpuscles of Tube 1. Washed antiserum-specific carbohydrate precipitate was then added to the serum from Tube 2, which was incubated for an hour. The precipitate was then centrifuged down, and the supernatant serum replaced on the washed corpuscles of Tube 2. There were thus two mixtures, one consisting of washed corpuscles and normal serum, the other consisting of washed corpuscles and serum which had been in contact with the precipitate for an hour at 37°C. A series of six tubes, each containing 0.5 cc. of the first mixture, was set up, similarly, a series of six tubes with the second mixture. Each series was inoculated with decreasing numbers of organisms in the usual way, the tubes were sealed, incubated in the rotating apparatus, and then plated out on the following day. Table IV shows the result of this experiment.

TABLE IV

Number of diplococci inoculated	Washed corpuscles + normal serum	Washed corpuscles + precipitate-treated serum
700,000	++++	++++
70,000	+++	+++
7,000	++	+
700	0	0
70	0	0
7	0	0

Thus it is seen that the precipitate does not appear to act on the serum in any way. By this process of exclusion, one is forced to conclude that the precipitate acts on the leucocytes themselves.

It is not definitely known how the precipitate prevents the leucocytes from effectively phagocytizing the sensitized pneumococci, but Cromwell and Centeno in a recent paper (4) have described the vacuolation of leucocytes when incubated with specific precipitates. These authors are of the opinion that the vacuolation is associated with digestion of the precipitate. Therefore, it may well be that such vacuolated leucocytes, while digesting the precipitate, are incapable of taking up sensitized pneumococci in the normal manner.

If the phagocytic function of the leucocytes is impaired by the digestion of the precipitate, it would of course follow that the interference

with bacterial phagocytosis is non-specific. That, for example, a Type III precipitate would interfere with the phagocytosis of the other types of pneumococci, as well as with Type III. That this is the case is shown by the following experiment, in which washed Type III precipitate was added to a series of tubes containing 0.5 cc. of blood, while an equal amount of saline solution was added to another series of tubes containing the same amount of blood. Each series was then inoculated with decreasing numbers of Type I organisms, the tubes sealed, incubated on the rotary apparatus, and plated out the next day. (See Table V.) The control blood in this experiment did not have a high phagocytic titre against Type I organisms, but in the presence of Type III precipitate, it had no titre at all against Type I pneumococci.

Up to this point, these phagocytic zones have been studied in

TABLE V

Number of Type I diplococci inoculated	Blood + Type III precipitate	Blood + saline
600,000	++++	++++
60,000	++++	++++
6,000	++++	+++
600	++++	0
60	++++	0
6	++++	0

relation to variations of the antiserum concentration, keeping the concentration of specific carbohydrate constant. But in the lungs, we have no knowledge of what the concentration of this substance may be. It can be shown that the zone of efficient phagocytosis moves slightly to the left if the concentration of specific carbohydrate is raised above that shown in Tables I and II, and that it moves slightly to the right if the concentration is lowered. It is probable that these shifts are bound up with the amount of precipitate formed with different concentrations of specific carbohydrate.

Although the experiments shown in Tables I and II are necessary to establish the phagocytic relationships between blood, specific soluble substance, antiserum and pneumococci, they are obviously much too extensive to carry out on every specimen of antiserum to ascertain the

optimum dilutions for *in vitro* phagocytosis. An endeavor was therefore made to cut such experiments down to a more reasonable size, without at the same time losing any essential information about the antiserum. This can be done satisfactorily by setting up a series of tubes containing a constant amount of blood, a constant amount of specific soluble substance, a constant number of pneumococci, and a varying amount of the antiserum to be tested. The following are the details of a Type I experiment set up in this way:

A series of tubes was filled with 0.5 cc. of blood; two drops of a 1/10,000 concentration of Type I specific soluble substance were added to each tube, giving a final concentration of about 1/75,000 of this substance; one drop of a 1/10 dilution of an 18 hour culture of Type I pneumococcus in rabbit blood hormone broth was also added to each tube (in the actual experiment shown in Table VI, the drop contained approximately 300,000 diplococci); and finally, a drop of serum was added to each tube, undiluted in the first tube, diluted 1/2 in the second tube, diluted 1/4 in the third tube and so on, giving final concentrations of the serum in the tubes as follows: 1/20 in the first tube, 1/40 in the second tube, 1/80 in the third tube, and so on. The tubes were sealed, incubated in the rotating machine and plated out the next day. In this experiment, the results of which are shown in Table VI, three different antisera were titrated in this way on two different specimens of human whole blood. Antiserum X was an unconcentrated serum containing 2000 units, Antiserum Y was a Felton concentrated serum containing 4500 units, Antiserum Z was a Felton concentrated serum containing 3500 units.

In general, these three antisera show the same end-point, except that Antiserum Z appears slightly stronger than the others with Blood A. But the two concentrated antisera have a definitely longer inhibition zone than the unconcentrated serum. This experiment shows approximately the maximum phagocytic action of the antiserum under these conditions; so that the zone of efficient phagocytosis is a comparatively narrow one. If fewer organisms were added to the tubes, this zone would of course be wider.

It would not be difficult to standardize antisera by this method, the standard antiserum being always set up under the same conditions as the unknown antisera, but until more is known of the significance of the phagocytic titre of an antiserum, a discussion of whether such a method is to be preferred to the mouse protection method would be premature. Theoretically, there are objections to both methods as a measure of the therapeutic value of an antiserum, and Goodner's

therapeutic method (5) of testing antiserum on infected rabbits would be the method of choice if one could be quite confident that the lung lesion in a human being reacted to antiserum like the skin lesion in a rabbit.

It is hardly necessary to say that if it could be shown that the *in vitro* phagocytic inhibition zone could be paralleled *in vivo* by a protection inhibition zone, the test-tube experiments would have practical importance, and could not be considered as of theoretical interest only. Protection inhibition zones in mice have been described by Felton

TABLE VI

Concentration of antiserum	Blood A			Blood B		
	Antiserum X	Antiserum Y	Antiserum Z	Antiserum X	Antiserum Y	Antiserum Z
1/20	++++	++++	++++	++++	++++	++++
1/40	++++	++++	++++	++++	++++	++++
1/80	++++	++++	++++	++++	++++	++++
1/160	+++	++++	+++	+++	++++	++++
1/320	+	+++	+++	+	+++	+++
1/640	0	+++	+++	0	+++	+++
1/1,280	0	0	0	0	+	0
1/2,500	0	0	0	0	0	0
1/5,000	0	0	0	0	0	0
1/10,000	0	0	0	0	0	0
1/20,000	++++	+++	0	+	+	+
1/40,000	++++	++++	++++	++++	++++	++++
1/80,000	++++	++++	++++	++++	++++	++++

and Bailey (6) and by Sobotka and Friedlander (7). It has not been possible to demonstrate a protection inhibition zone in Type I experiments on mice, using 0.2 cc. of undiluted 2000 unit antiserum as the maximum dose of serum. In the case of Type III, however, more success was obtained, as the following experiment shows:

Forty-two mice were divided into seven groups of six mice each. All the mice received an injection of 0.2 cc. of a 1/10,000 concentration of Type III specific soluble substance. Group I received 0.2 cc. of undiluted Type III antiserum; Group II 0.2 cc. of a 1/4 dilution of the antiserum; Group III 0.2 cc. of a 1/16 dilution; Group IV 0.2 cc. of a 1/64 dilution; Group V 0.2 cc. of a 1/256 dilution; Group VI 0.2 cc. of a 1/1000 dilution; Group VII 0.2 cc. of a 1/4000 dilution.

One mouse in each group was then injected with 0.5 cc. of a 1/5 dilution in broth of an 18 hour broth culture of Type III pneumococcus—the minimal lethal dose of the culture being 0.000,000,1 cc.; one mouse in each group was injected with 0.5 cc. of a 1/500 dilution of culture, and so on, so that the sixth mouse in each group received 0.5 cc. of a 1/500,000 dilution of the culture. Table VII shows which mice had died and which survived at the end of 96 hours.

Owing doubtless to the difference in resistance between one mouse and another, the result of this *in vivo* experiment is not as consistent as the parallel *in vitro* experiment shown in Table I, but the general picture is clear enough—a well-marked protection inhibition zone with undi-

TABLE VII  
*0.2 Cc. of 1/10,000 Concentration of Specific Soluble Substance*

Dilution of culture	0.2 cc. of undiluted antiserum	0.2 cc. of 1/4 dilution antiserum	0.2 cc. of 1/16 dilution antiserum	0.2 cc. of 1/64 dilution antiserum	0.2 cc. of 1/256 dilution antiserum	0.2 cc. of 1/1,000 dilution antiserum	0.2 cc. of 1/4,000 dilution antiserum
1/5	D	D	D	D	D	D	D
1/50	D	D	D	S	S	D	D
1/500	S	S	S	S	S	D	D
1/5,000	D	D	D	S	S	D	D
1/50,000	D	S	D	S	S	S	D
1/500,000	S	D	S	S	S	S	S

S = survival.

D = death.

luted serum, with serum diluted 1/4 and 1/16; a zone of efficient protection with serum diluted 1/64 and 1/256; followed by a zone of ineffective protection when the serum was diluted too highly. The experiment is sufficiently clean cut. It suggests that if the injection of Type III specific soluble substance into a mouse at the same time that it is infected with Type III pneumococci parallels the case of a human being suffering from Type III pneumonia, then a comparatively small dose of Type III antiserum is all that is necessary to effect a cure, while more than that amount of serum would be harmful.

If it were possible to find an animal which reacted with a localized lesion to pneumococcus infection, and whose blood reacted similarly to human blood, it would be possible to carry out controlled therapeu-

tic experiments with different amounts of antiserum, based on the *in vitro* measurements. In the attempts that have been made, rabbit blood has not reacted like human blood in the presence of antiserum, specific soluble substance and pneumococci.

An opportunity arose, however, through the courtesy of Doctors Sutliff and Finland of the Boston City Hospital, to test the blood of a case of Type I pneumonia which was being treated with large doses of strong antiserum.

A specimen of blood was taken from the patient after 28 cc. of antiserum had been administered, and again 14 hours later, after 180 cc. of antiserum had been given in all. On the theory that the rôle of the antiserum is to neutralize the specific soluble substance in the lung, each specimen of blood was divided into

TABLE VIII

Number of diplococci inoculated	Blood after 28 cc. of antiserum				Blood after 180 cc. of antiserum			
	Concentration of spec. sol. sub.							
	1/1,600	1/16,000	1/160,000	0	1/1,600	1/16,000	1/160,000	0
350,000	++	+	+	0	++	++++	++++	0
35,000	0	++	0	0	+++	+++	+++	0
3,500	0	0	0	0	++	++	++	0
350	0	0	0	0	++	++	++	0
35	0	0	0	0	+	+++	+++	0
3	0	0	0	0	0	0	0	0

parts; one part was kept as a control and a varying amount of the specific carbohydrate was added to each of the other parts. All were then tested in the usual way by infecting with Type I pneumococci, incubating, and plating out. Table VIII shows the result of this experiment.

The table shows at a glance that phagocytosis is far more effective under these conditions after a dose of 28 cc. of antiserum than after 180 cc., no matter what concentration of specific soluble substance is present. A simple calculation, based on a blood volume of 5000 cc., shows that a dose of 28 cc. of antiserum results in a concentration of 1/180 of antiserum in the patient's blood, and that a dose of 180 cc. of antiserum results in a concentration of 1/28 of antiserum. Reference to Table II shows that with a concentration of



1/180 of antiserum, phagocytosis is fairly good, while with a concentration of 1/28 of antiserum, phagocytosis is very markedly inhibited. So the results obtained by the *in vitro* addition of the antiserum (shown in Table II) parallel fairly well the results with the *in vivo* injection of the antiserum (shown in Table VIII). To obtain the optimum phagocytic effect in the presence of specific soluble substance, probably the injection of only 10 cc. of the antiserum would have been sufficient in the above case. It is significant that 2 hours after 28 cc. of antiserum had been administered, there were still pneumococci in this patient's blood stream, probably thrown off from the lesion, because it will be seen from the table that the blood alone was strongly phagocytic. But after 180 cc. of serum had been given, the blood stream was sterile, and the patient's general condition improved. This improvement was maintained and recovery was uninterrupted. In other words, a dose of antiserum which was fairly efficient in promoting phagocytosis in the presence of the specific carbohydrate led to no apparent clinical improvement, whereas a much larger dose of antiserum which resulted in little or no phagocytosis in the presence of the carbohydrate, led to apparently marked clinical improvement.

A Type III antiserum was titrated out for its opsonic power in the presence of Type III specific soluble substance. It was a weak serum and showed its optimum phagocytic effect in a concentration of 1/80. To attain this concentration in the blood of an adult, about 60 cc. would have to be given. This dose was injected, but had no apparent apparent clinical effect whatever on the Type III cases in which it was tried.

#### DISCUSSION

It is well-known that *in vitro* phagocytosis of virulent pneumococci by human blood is aided by the addition of antiserum alone; Sia's work has been confirmed that phagocytosis is hindered by the addition of specific carbohydrate alone; and it has been demonstrated in these experiments that when both antiserum and specific carbohydrate are present, phagocytosis may be inefficient if there is too much antiserum as well as too little antiserum. There is, however, an optimum concentration of antiserum which will neutralize the specific carbohydrate and aid phagocytosis. This optimum concentration differs in different antisera, and must be determined by testing each antiserum.

The inhibition of phagocytosis, when strong antiserum is used to neutralize the specific carbohydrate is probably caused by the specific precipitate formed interfering, perhaps mechanically, with the ingestion of the pneumococci by the leucocytes. The phagocytic inhibition zone is better marked with Type III pneumococcus than with Type I, probably because the Type III precipitate is much heavier than the Type I precipitate. That the interference with phagocytosis by precipitates is a general phenomenon is borne out by the work of Todd (8), who has described the inhibiting action of precipitates in the phagocytosis of staphylococci and streptococci.

It is an open question what bearing these results have on what occurs in the lungs in cases of pneumonia specifically treated with antiserum. For some years now it has been generally thought that the rôle of the antiserum is not simply one of sensitizing the invading pneumococci to phagocytosis. Patients suffering from pneumonia were found often times to have in the urine a substance which reacted specifically with the antiserum—the “specific soluble substance” of Dochez and Avery, the “residue antigen” of Zinsser. Since that time, the antiserum has been assigned at least two functions, firstly of neutralizing the specific soluble substance, and secondly of sensitizing the pneumococci. It was further believed, quite logically, that if the specific soluble substance remained in excess, the pneumococci would not be sensitized, because the same antibody in the antiserum was concerned in neutralization of the specific soluble substance and the sensitization of the pneumococci. In other words, this theory demanded that there had to be enough of this antibody remaining in excess after the specific soluble substance had been neutralized, to sensitize the organisms. Success in specific therapy therefore depended on giving enough antiserum to more than neutralize the specific soluble substance, and the large doses of antiserum that were apparently necessary were accounted for by the difficulty of this neutralization, and in the case of Type III pneumonia, this was almost if not quite impossible with the antisera available.

In the experiments described in this paper, an attempt has been made to study the quantitative relationship between antiserum, specific soluble substance and living pneumococci by a phagocytic technique in test-tubes, using human whole blood. It has been found that in

this comparatively simple phagocytic system, a surprisingly small concentration of therapeutic antiserum is sufficient to neutralize moderately strong concentrations of specific soluble substance and still leave enough antiserum in excess to sensitize the pneumococci. And further that if the concentration of the antiserum rose to the concentration generally used in therapy, an inhibition zone very markedly reduced the amount of phagocytosis. Despite the disappointing results of Type III therapy, it was found to be just as easy to neutralize the Type III specific soluble substance as it was the Type I substance. Naturally the first deduction made in the light of these experiments was that the dose of antiserum in treating pneumonia was far too large. This theory was put to the test and found to be erroneous. In an actual case of Type I pneumonia treated with antiserum in which the blood was tested at two stages during the treatment, it was found that after a fairly small dose of antiserum, the phagocytic titre of the blood in the presence of the specific carbohydrate was good, but no clinical effect could be observed, the blood culture remaining positive; whereas after a fairly large dose of serum had been administered, the phagocytic titre of the blood had almost vanished in the presence of the carbohydrate, but the clinical effect was a definite improvement, which was subsequently sustained. Further, a dose of Type III antiserum, calculated to effectively neutralize the Type III specific soluble substance and promote optimum phagocytosis, had no clinical effect whatever when injected into cases of Type III pneumonia.

What then is the explanation of this contradiction? No definite answer can be given at the present time, but there are several explanations possible.

It might be said that one cannot argue from an artificial and admittedly incomplete combination of reacting substances in test-tubes to the actual processes in the lesion. While it is true that one cannot argue definitely from one to the other, this test-tube method, however imperfect, is perhaps the most direct approach available towards the understanding of the protective mechanism in pneumonia. The results, though not conclusive, may well be suggestive, even if they imply a contradiction to the present theories.

It might be said that—granted that only a minimal amount of antiserum is necessary to neutralize the specific soluble substance—the circulation in the lesion is so poor that the antiserum does not reach the infected tissues in the concentration in which it is present in the blood stream. However, a reference to Table II shows that if a concentration of antiserum is reached which is just on the edge of the inhibition zone—as was the case in the above mentioned patient after the fairly small dose of antiserum had been given—the concentration of the antiserum can fall considerably without any marked reduction of phagocytosis.

A third possible explanation is that there is another substance present in the lesion, other than the specific soluble substance, which has to be neutralized before the pneumococci can be sensitized and phagocytosed, and that a high concentration of antiserum is necessary to effect this. It is in this direction that this study is being extended.

In the mouse experiments, no explanation can be offered as to why it was possible to show a marked inhibition zone in the protective action of Type III antiserum in the presence of the specific carbohydrate and impossible to show a zone in similar experiments with Type I antiserum, other than the heavier specific precipitate and the more marked phagocytic inhibition zone with Type III. This very simple explanation of the protection inhibition zone is tentative, and much further work is necessary to determine the relationship between the antiphagocytic effect of the precipitate and the failure of the strong antiserum to protect in the presence of the specific soluble substance.

#### CONCLUSIONS

1. *In vitro* phagocytic experiments with human blood, antipneumococcus serum, pneumococcus specific soluble substance, and living virulent pneumococci show that there is a definite phagocytic inhibition zone when strong antiserum is used.

2. If the antiserum is further diluted, there is a zone where phagocytosis is effective. If the serum is diluted still more, phagocytosis gradually falls off, as the very dilute antiserum fails to neutralize the specific carbohydrate, which has a specific antiphagocytic action.

3. The inhibition zone is apparently caused by the specific precipitate (formed by the antiserum and the specific carbohydrate) interfering, perhaps mechanically, with the ingestion of the pneumococci by the leucocytes.

4. The inhibition zone is better marked with Type III than with Type I pneumococcus.

5. As the concentration of antiserum in the zone of effective phagocytosis *in vitro* does not correspond with the concentration of antiserum generally used *in vivo* in the serum therapy of pneumonia, this question is discussed.

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## THE TRANSMISSION OF YELLOW FEVER

EXPERIMENTS WITH THE "WOOLLY MONKEY" (*LAGOTHRIX LAGOTRICHIA* HUMBERT), THE "SPIDER MONKEY" (*ATELEUS ATER* F. CUVIER), AND THE "SQUIRREL MONKEY" (*SAIMIRI SCIREUS* LINNAEUS)\*

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### PLATE 12

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In two previous publications (1, 2) from this laboratory it has been shown that certain *Cebus* monkeys can be infected with yellow fever virus but that the manifestations of disease are usually mild compared with those in *Macacus rhesus*. From time to time it has been possible to obtain specimens of other South American monkeys for experimentation.

#### *Experiments with Lagothrix lagotricha Humboldt*

The Humboldt monkey, "woolly monkey," or "barrigudo," is a gentle creature but, unfortunately, it is not hardy. Many live only a short time in captivity. It is difficult to arrange a diet upon which they can thrive.

Attempts have been made to infect twelve young animals of this species with yellow fever virus. In three instances a fever has followed inoculation. *Lagothrix* 2 had a temperature of approximately 102° when on April 2, 1929, it was inoculated with Asibi strain virus. On April 17 the temperature reached 104.8°

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and blood was transferred to *Lagothrix* 3. On April 18 the temperature of No. 2 was still 104°, but it fell thereafter to normal and later to subnormal (96°). The animal was sacrificed on April 30; the autopsy showed nothing suggestive of yellow fever. *Lagothrix* 3, inoculated on April 17, had a temperature of 105.8° on April 20; there was fever also on the following 2 days. On April 20 blood was transferred to *Lagothrix* 5 and mosquito Batch 177 was allowed to feed. No. 5 did not react. On May 8 Batch 177 fed on *Rhesus* J1. The latter showed no fever during 16 days of observation; it was then given an immunity test but continued afebrile. The control for the immunity test, *Rhesus* J2, died with yellow fever. On May 2 *Lagothrix* 3 was moribund; he was bled and sacrificed. There were no lesions suggestive of yellow fever. Tests with sera in 3 cc. amounts from No. 2, taken 12 days after the last febrile access, and from No. 3, taken 9 days after the last fever, showed protection against virus. Serum from No. 2 also protected in a dose of 2 cc., but a like quantity from No. 3 failed; the control which received normal *Lagothrix* serum died on the third day.

On July 27, 1929, *Lagothrix* 11 was inoculated with citrated blood containing virus of the Asibi strain. On August 8 the temperature reached 106°, and blood was transferred to *Rhesus* J10. The latter showed a slight fever on August 12 and for 6 days thereafter. The maximum temperature was 104.8°. On September 13 he was given a test dose of virus, which he survived without showing fever.

*Ateles ater* 2 was injected subcutaneously with infectious liver emulsion, containing Asibi strain virus, on May 29, 1929. The temperature of the animal at that time was 102.9°. On June 3 the temperature had risen to 103.8° and mosquito Batch 207 was allowed to feed; the same batch was applied on June 4, but probably very few mosquitoes fed at that time. On June 7 the temperature of No. 2 had dropped to 100.9°; the animal had no appetite, was weak and obviously ill. He was sacrificed, but no gross lesions suggestive of yellow fever were found. Microscopically the liver appeared uninjured and the parenchyma was stocked with glycogen. The kidneys showed a slight cloudy swelling, and two focal abscesses were noted in the section examined. Liver emulsion from *A. ater* 2 was inoculated into *Rhesus* J3 and caused the death of the animal on June 15, the eighth day after inoculation. The gross and microscopic pictures were typical of yellow fever. Mosquito Batch 207 was allowed to feed on *Rhesus* J7 on July 4. The animal died on July 9 with typical yellow fever.

None of the other "woolly monkeys" have exhibited any reaction to yellow fever virus. However, it is only fair to state that three died from undetermined causes within a week after inoculation. Protection tests have been made with sera from *Lagothrix* Nos. 6, 7, 11, and 12. *Rhesus* monkeys were protected by serum from the last three. Serum from No. 6 failed to protect; this animal had been fed upon by mosquitoes, but never showed the slightest reaction. *Lagothrix* 11 has

already been discussed. *Lagothrix* Nos. 7 and 12 had been inoculated with virulent blood from animals experimentally infected with the Asibi strain, but they had not shown a febrile reaction. However, serum from No. 7 gave perfect protection to *Rhesus* J8, while that from No. 12 enabled *Rhesus* J13 to survive.

### *Experiments with Ateles ater F. Cuvier*

The spider, or "aranha," monkey, is a long-legged, ungainly creature, but gentle and not difficult to handle. It lives well in captivity. Four specimens have been used in the course of our experiments. Nos. 1 and 4 were inoculated with infectious blood, Asibi strain, but did not show a temperature reaction. However, the serum of No. 4, taken 14 days after inoculation, protected a *rhesus* monkey against virus (see Table III).

*Ateles ater* 3 was fed upon, September 16, 1929, by two batches of mosquitoes infected with the S.R. strain. On September 20 and 21 the animal showed fever; the highest temperature noted was 104.5°. *M. rhesus* J14 was injected with 6 cc. of blood on September 20. He died on September 25 with typical gross and microscopic lesions of yellow fever. Mosquito Batch 249 was also allowed to feed on September 20. On September 23 *A. ater* 3 could not stand up, because of tetany in the limbs. It was found dead on the morning of September 24. The lobule peripheries of the liver were paler than normal, but the organ did not approach in color the typical boxwood of yellow fever. There was a faint icterus discernible in the intima of the aorta. There were a few brownish streaks, probably of changed blood, on the gastric mucosa. Bladder urine contained 0.3 gm. of albumin per liter. Other findings at autopsy appeared to have no relation to a possible yellow fever. The microscopic examination revealed no necrosis in the liver; there was a little fat around the portal spaces and a moderate engorgement of the vessels; a few leucocytes were present and the Kupffer cells were loaded with clumps of dark pigment (the blood had not been examined for malarial parasites). The kidneys showed a moderate cloudy swelling and some congestion of the vessels.

On October 10, mosquito Batch 249 (engorged on *A. ater* 3, September 20) fed on *Rhesus* J18. Subsequently the monkey had fever for 2 days, but survived. On October 26 the same batch fed on *Rhesus* J20. The animal died with typical yellow fever on November 2.

### *Experiments with Saimiri sciureus Linnaeus*

The squirrel monkey or "macaco cheiro," is but little larger than the marmoset and almost as active. Of the Brazilian species thus far used



for experimentation it seems to be the most easily infected and is the only one which shows hepatic necrosis with any regularity.

Up to the present time thirty *Saimiri* monkeys have been either fed upon by infected mosquitoes or inoculated with blood or liver from animals thought to be infected. Not all have reacted, and of those that died or showed fever, not every one has been proved to have had yellow fever. Table I lists those animals which showed suggestive lesions at autopsy, those from which positive transfers were made to *Macacus rhesus*, and those whose sera after recovery gave protection against yellow fever virus.

Only a limited number of protocols will be given in full. Supplementary data will be found in the accompanying charts, tables, and schematic representations of transfers.

*Saimiri* 3 was inoculated intraperitoneally on March 8, 1929, with 4 cc. of citrated blood, containing Asibi strain virus, from *M. rhesus* B2. On March 8 and 9 the temperature ranged from 102° to 102.7°; on March 12 it reached 103.9° and in the morning of March 13 it was 104°. On both of the latter days blood was transferred to *Saimiri* 4. *Saimiri* 3 was found dead on March 14. At autopsy jaundice was doubtful. The heart and lungs were negative. The liver in general was pale; the lobule centers appeared darker, with yellow (fatty) peripheries. The spleen seemed normal. The kidneys were much injected, with a trace of subcapsular hemorrhage. The stomach contained changed blood mixed with food. Microscopic examination showed the liver architecture completely disorganized. There was a severe necrosis, irregularly affecting the midzones. Leucocytes, both polymorphonuclear and mononuclear, were present in great numbers. There was congestion of all blood spaces, and a little diffuse fatty infiltration (Figs. 1 and 2). The kidneys showed injection of vessels and a suggestion of cloudy swelling.

*Saimiri* 13 was fed upon May 16, 1929, by mosquito Batch 154, infected with the Asibi strain. The initial temperature was 102.6°. On May 18 the temperature reached 103.9°, on May 19 it was 104.2°, and on the following day 105°. There was fever until May 22, again on May 24, and from May 27 to 30. On 3 successive days, May 18, 19, and 20, blood was transferred to *Rhesus* R6 and *Saimiri* 14; mosquito Batch 195 was allowed to engorge on these same days. *M. rhesus* R6 died on May 24 with typical yellow fever. Mosquito Batch 195 was applied to *Rhesus* J5 on June 12; the animal showed no febrile reaction, but later survived its immunity test. On June 19 the mosquitoes were injected subcutaneously into *Rhesus* J6; the latter had fever for 3 days and was killed when moribund with yellow fever, on June 27. *Saimiri* 13 was bled for a protection test on June 7 and on the following day was given a test dose of virus. On June 10 and 12 the temperature reached 104° and blood was transferred to *Rhesus* J4 upon two occasions. The *rhesus* showed no reaction. On June 22, No. 13 was bled again. The results of protection tests are indicated in Table III.

*Saimiri* 16 was fed upon on July 12, 1929, by mosquito Batches 199 and 212, infected with the S.R. strain. On July 16 its temperature reached 104° and blood was transferred to *Rhesus* J9 and *Saimiri* 17; mosquito Batch 224 was allowed to

TABLE I  
*Yellow Fever Infections in Saimiri sciureus*

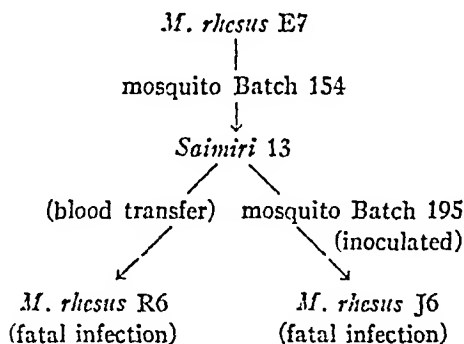
<i>Saimiri</i> No.	Mode of infection	Virus strain	Out- come	Criteria of infection
1	Inoculation of blood	Asibi	D	Necrosis and fat in liver. Cloudy swelling in kidneys.
2	"	"	D	Liver necrosis and disorganization. Cloudy swelling in kidneys.
3	"	"	D	Febrile reaction. Gastric hemorrhage. Severe liver necrosis.
5	"	"	D	Febrile reaction. Virus transferred back to <i>rhesus</i> .
6	Mosquito feeding	"	R	Febrile reaction. Serum gave positive protection test. Mosquitoes fed on No. 6 produced immunity in <i>rhesus</i> monkey.
9	Inoculation of blood	"	R	Transfer from No. 6. Serum gave positive protection test.
13	Mosquito feeding	"	R	Febrile reaction. Virus transferred back to <i>rhesus</i> by blood inoculation and by mosquitoes. Serum gave positive protection test.
16	"	S.R.	D	Febrile reaction. Gastric hemorrhage. Liver necrosis. Virus transferred back to <i>rhesus</i> by blood inoculation and by mosquito feeding.
18	Inoculation of blood	Asibi	D	Liver necrosis. Cloudy swelling and many casts in kidneys.
20	"	"	R	Febrile reaction. Serum protective.
22	Mosquito feeding	"	D	Febrile reaction. Virus transferred back to <i>rhesus</i> by blood inoculation and by mosquitoes. Liver necrosis.
23	"	S.R.	R	Febrile reaction. Virus transferred back to <i>rhesus</i> .
25	Inoculation of blood	"	S	(Transfer from No. 23.) Febrile reaction. Liver necrosis.
27	Inoculation of liver emulsion	"	D	(Transfer from No. 25.) Febrile reaction. Liver necrosis.
28	Inoculation of blood	"	D	(Transfer from No. 27.) Liver necrosis.

D = died. R = recovered. S = sacrificed.

feed. On July 17 the temperature of No. 16 dropped to 94°, and it was sacrificed when moribund. There was a suggestion of icterus in the tarsal plates and in the intima of the aorta. The liver was friable, very pale, and fatty. The stomach contained a small amount of definite black vomit. The other organs did not

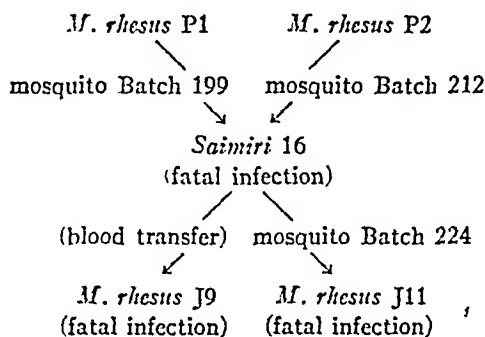
*Scheme I*

(Asibi strain)



*Scheme II*

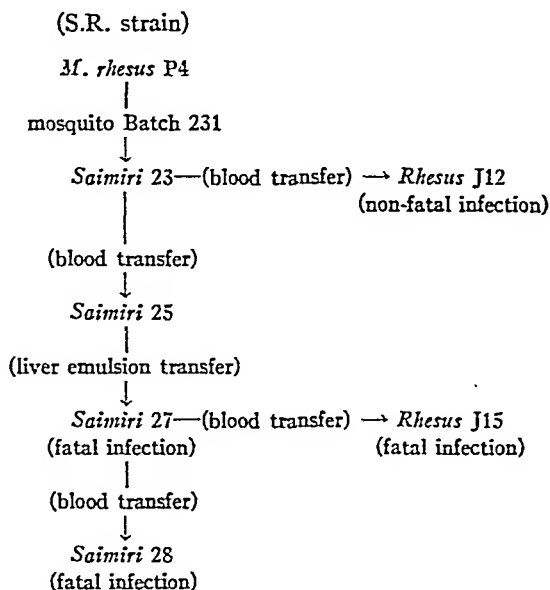
(S.R. strain)



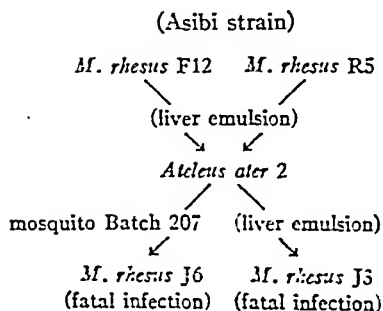
appear grossly abnormal. Microscopic sections showed the liver columns to be jumbled. There was severe necrosis, more especially in the mid-zones, but even here there were occasional fairly normal nuclei to be found. There appeared to have been considerable removal of necrotic material. There was a great invasion of leucocytes, principally polymorphonuclears. Congestion was general and

small hemorrhages quite numerous. The kidneys showed congestion and a mild cloudy swelling. *M. rhesus* J9, which had received blood from this *Saimiri*, died with typical yellow fever on July 20. Mosquito Batch 224 fed on *Rhesus* J11 on

### Scheme III

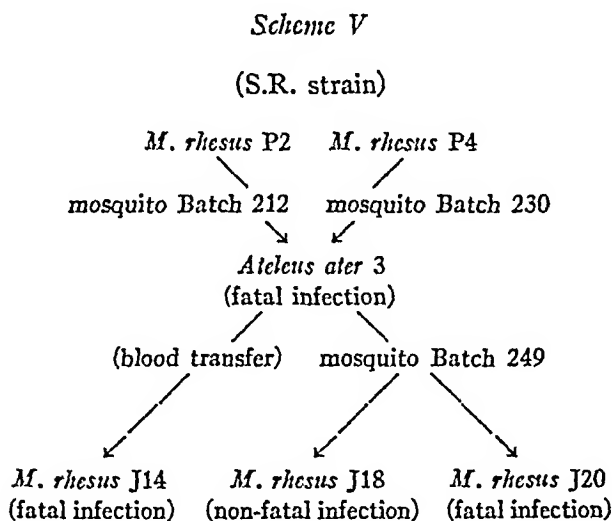


### Scheme IV



September 10; the animal was found dead on September 15. The lesions were typical of yellow fever.

*Saimiri* 23 was fed upon September 9, 1929, by mosquito Batch 231, infected with the S.R. strain. On September 13 the temperature reached 104.4° and on September 14 it was 104°. Blood was transferred to *Saimiri* 25 and to *Rhesus* J12. The latter had fever on the sixth and seventh days after inoculation, but recovered. No. 25 had fever on September 22, 23, and 24, the maximum being 104.7°. On September 24 the animal was killed and a transfer of liver emulsion made to *Saimiri* 27. No. 25 had a definite icterus in the tarsal plates. The liver was extremely friable; the general color of this organ was orange, with certain areas apparently hemorrhagic; the normal markings were obliterated. The stomach contained a food bolus smeared with changed blood. Microscopically, the liver



showed severe necrosis, midzonal, but usually extending more toward the portal spaces; there were a trace of fat present and a great many leucocytes. The kidneys showed a little cloudy swelling, with a few early casts.

*Saimiri* 27, inoculated with liver from No. 25 on September 24, had a temperature of 104.1° in the afternoon of the 26th. On September 27 the morning temperature was 104.5° and the afternoon temperature 94.5°. Blood was transferred to *Saimiri* 28 and to *Rhesus* J15. No. 27 died during the night of September 27-28. None of the organs were remarkable except the liver, which was very friable and of a uniform yellowish-gray color. Microscopically, the liver showed some necrotic cells, especially in the mid-zones, and a heavy, widespread infiltration of fat in large globules; congestion was moderate. Kidney changes were thought to be largely postmortem. *M. rhesus* J15, inoculated on September 27, had a fever of 105.4° on October 3. On October 4 the morning temperature was 104.8° and the

afternoon temperature 101.9°. The animal was very ill and was sacrificed; the autopsy revealed gross and microscopic lesions of yellow fever.

*Saimiri* 28 had a temperature of 102° when inoculated; the maximum thereafter was 103.6°, but the animal was found dead on October 3. There was a definite icterus of tarsal plates and intima of aorta. The liver was very friable; it was the

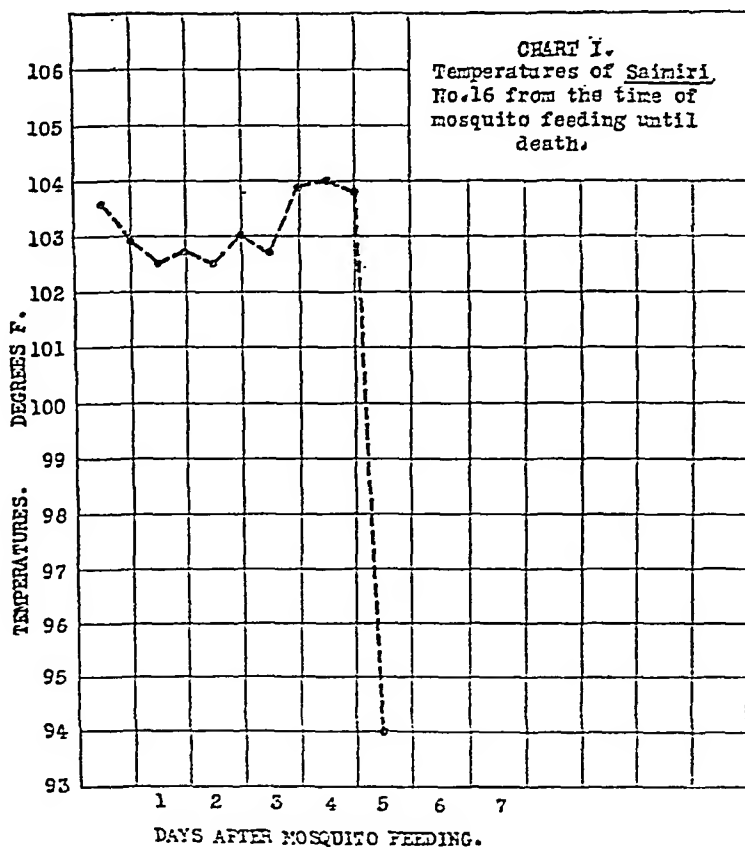


CHART I

color of putty except for a little superficial mottling. The kidneys were swollen, the cortices opaque, the vessels injected. The stomach contained a little changed blood. Microscopically the liver showed only a few cells approaching normal in the portal spaces. Necrosis was very extensive; the involved parenchyma showed disintegration of cytoplasm, karyorrhexis of nuclei, etc. There were congestion,

infiltration of fat, and invasion of leucocytes. The kidneys showed postmortem changes.

*Saimiri* 23 was given an immunity test with Asibi strain virus on September 28, a fortnight after the last fever. On October 3 the temperature rose to 104.2° and blood was transferred to *Rhesus* J16. The latter never reacted. Serum from

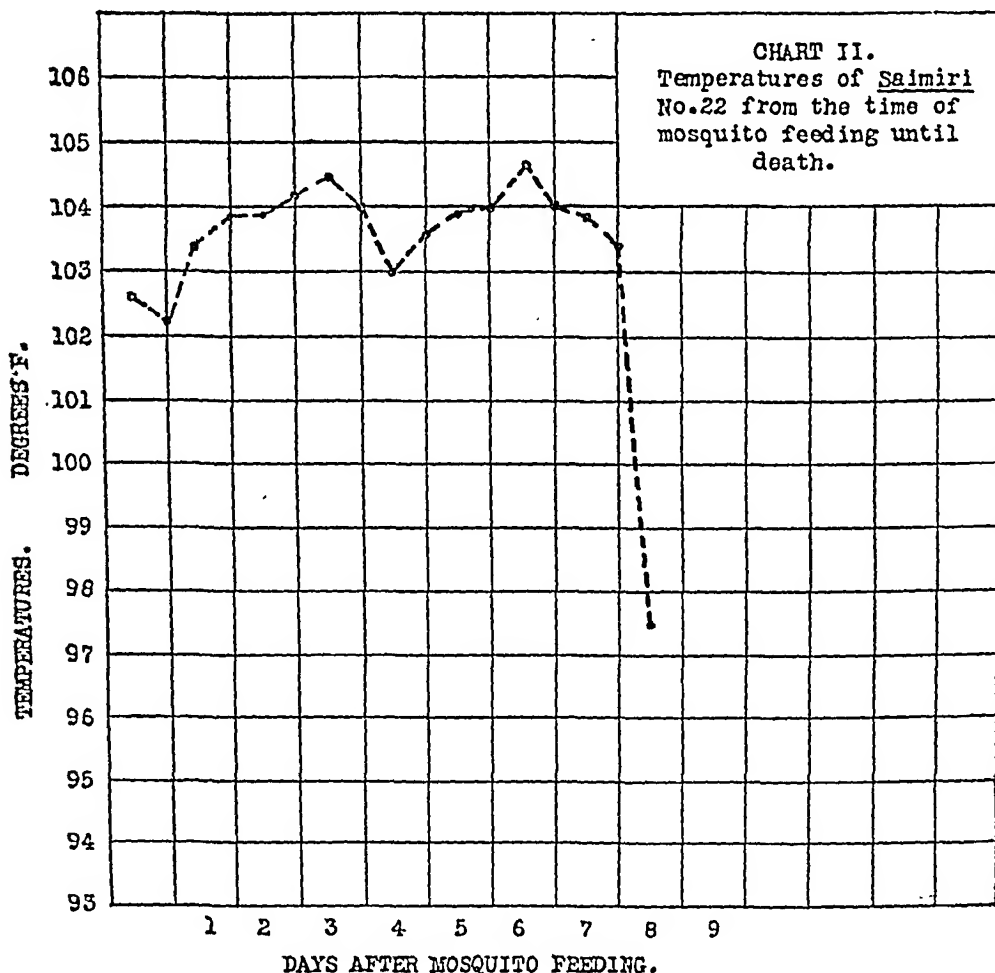


CHART II

No. 23 proved to be highly protective against yellow fever virus (*Rhesus* J19, Table III).

### Pathology

Although two of the spider monkeys (*Ateles ater*) became exceedingly ill following the introduction of yellow fever virus, the lesions at

autopsy were not comparable to those found in human beings and in *rhesus* monkeys which have died with the disease.

A considerable number of *Saimiri* monkeys have succumbed to

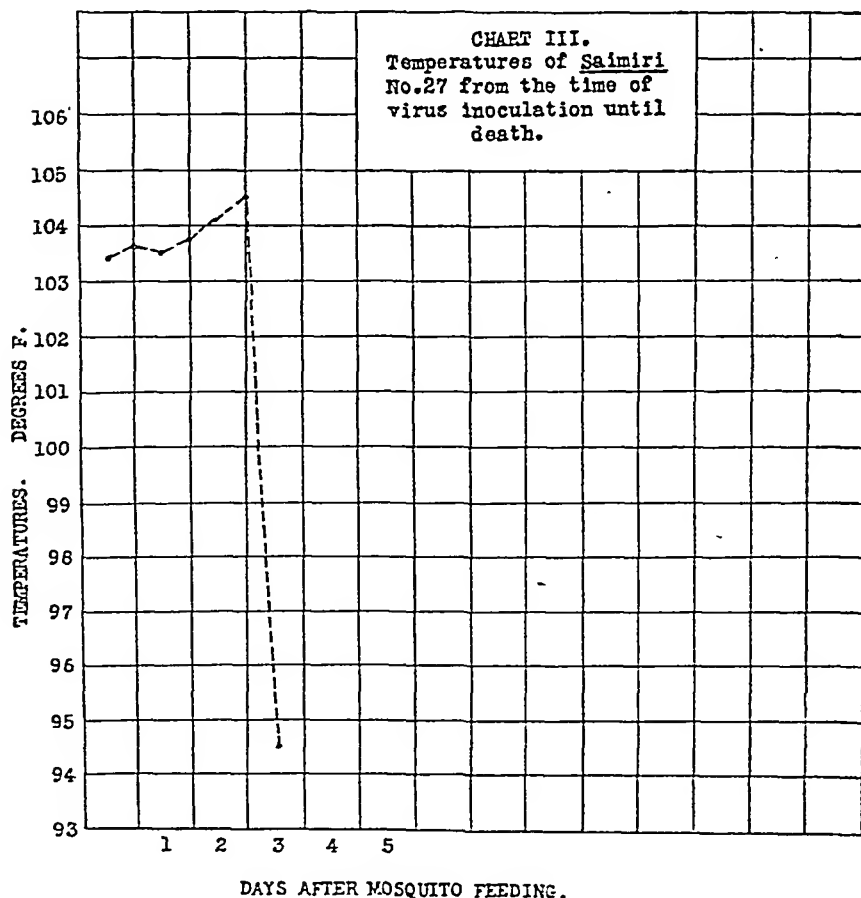


CHART III

yellow fever infections. In some of these fatal cases the gross appearances at autopsy were quite typical of yellow fever.

The liver was orange, yellow, or grayish in color, obviously fatty, and very friable. At several autopsies changed blood was found in the stomach. Icterus



TABLE II  
*Temperature Reactions of Infected Saimiri sciureus*

Saimiri No.	Source of infection		Strain	Temperatures A.M. and P.M.												
	Blood or tissues from	Mosquito Hatch No.		Initial day	1	2	3	4	5	6	7	8	9	10	11	12
1	<i>Rhesus</i> P13 and P6	—	Asibi	—	102.0 102.6	102.6 102.8	102.4 102.1	103.8 103.7	103.5 103.6	103.2 103.0	102.8 103.7	103.4 103.2	97.2 S			
2	<i>Rhesus</i> B2	—	"	103.0 102.7	101.9 102.0	102.1 103.7	103.5 103.0	102.6 102.4	D —							
3	<i>Rhesus</i> B2	—	"	102.7 102.0	102.1 102.5	103.0 103.8	103.7 103.6	103.4 103.9	104.0 103.0	D —						
5	<i>Rhesus</i> E7	—	"	103.8 103.7	103.8 103.9	104.2 102.0	103.7 102.0	101.8 102.1	102.6 102.7	102.4 102.0	97.2 100.0	98.6 100.0	D —			
6	—	135	"	—	101.9 102.2	102.5 102.8	102.4 102.4	101.8 102.0	102.1 102.3	102.4 102.5	100.2 101.2	100.8 101.9	102.9 103.2	103.4 104.6	104.0 103.7	103.2 101.8*
13	—	154	"	—	102.0 102.6	103.9 102.6	103.4 104.2	104.4 105.0	104.8 104.5	104.2 104.0	103.9 103.8	104.6 104.0	103.9 103.6	103.5 —	104.2 104.0	104.4 104.2*
14	<i>Saimiri</i> 13	—	"	—	103.2 103.7	102.4 103.0	103.7 104.2	D —								
16	—	199 & 212	S.R.	—	102.9 103.6	102.7 102.6	103.0 102.8	103.9 104.0	103.8 94.0S							

18	<i>Rhesus</i> P8	—	Asibi	—	102.0 103.1	102.7 103.4	D	—									
20	<i>Rhesus</i> P7 and P8	—	"	—	103.0 102.6	103.6 103.5	103.7 103.9	103.6 103.8	103.4 103.2	103.6 103.5	103.4 103.2	103.0 102.9	102.6 103.0	103.5 103.4	103.6 103.5	103.4 103.5	103.7 103.9
22	—	223	"	—	102.3 102.6	103.8 103.4	104.2 104.4	104.0 103.9	103.6 103.9	104.0 103.0	103.6 103.9	104.0 104.5	104.0 103.9	103.6 97.6S			103.9 104.4*
23	—	231	S.R.	—	103.8 102.9	103.8 103.7	103.7 103.6	103.8 103.9	104.0 103.9	103.8 104.4	104.0 103.9	103.6 103.7	103.5 103.6	103.4 103.5	103.2 102.8	103.5 103.4	103.5 103.2
25	<i>Saimiri</i> 23	—	"	—	102.8 102.4	103.0 103.5	103.4 103.5	103.2 103.0	103.0 102.6	102.9 102.9	103.0 102.9	103.4 103.5	103.7 103.6	103.8 104.6	104.2 104.7	104.2 S	
27	<i>Saimiri</i> 25	—	"	—	103.6 103.4	103.7 103.5	104.5 104.1	104.5 94.5	D	—							
28	<i>Saimiri</i> 27	—	"	—	102.0 101.8	102.9 103.2	103.6 103.5	102.1 102.6	102.6 103.4	102.9 103.0	102.6 103.4	D	—				

\* Recovered. D = died spontaneously. S = sacrificed.

TABLE III  
*Immunity Tests with Sera from South American Monkeys*

Experi- ment No.	Test animal M. rhesus No.	Test strain of virus	Brazilian animal tested	Original infection strain	Amount of serum used	Interval between in- roduction of virus and withdrawal of serum	Interval between last fever and withdrawal of serum	Reaction of <i>rhesus</i> to test			
								Length of fever	Maxi- mum tempera- ture	Out- come	Day of death (from initiation of expt.)
I	L1	Asibi	Normal Saimiri (Control)	—	cc.	days	days	days	°F.		
	L2	"	Saimiri 10	Asibi	2	—	—	2	104.4	D	4
	L5	"	Saimiri 6	"	2	17	16	1	104.8	D	4
	L3	"	Lagolhriz 3	"	2	27	16	5	104.9	R	—
	L4	"	Lagolhriz 2	"	2	15	9	1	104.2	D	4
II***	L10	"	Normal Lagolhriz (Con- trol)	—	2.7	28	12	2	104.1	R	—
	L11	"	Lagolhriz 3	Asibi	3	—	—	1	104.2	D	3
	L12	"	Lagolhriz 2	"	3	15	9	5	105.2	R	—
	J8	"	Lagolhriz 7	"	3	28	12	4	105.0	R	—
	L9	"	Lagolhriz 6	"	3	14	No fever	—	103.5	R	—
	L8	"	Saimiri 9	"	1.8	15	No fever	1	104.7	D	4
	L7	"	Saimiri 13	"	2	20	No fever	7	105.0	R	—
	L6	"	Saimiri 13	"	3	22*	8	10	105.0	R	—
						{ 39	10	5	104.3	R	—
						{ 14**					
III	L13	"	Normal Saimiri (Control)	—	3	—	—	1	105.6	D	4
	L14	"	Aotus 1	Asibi	3	17	No fever	5	105.2	R	—
	L15	"	Lagolhriz 12	"	3	17	No fever	7	105.0	R	—
	L16	"	Lagolhriz 11	"	3	27	14	—	103.7	R	—
	L17	"	Saimiri 17	S.R.	3	15-23	No fever	3	104.9	D	8
	L18	"	Saimiri 20	Asibi	3	31	14	7	104.7	R	—
	M5	"	—	—	No serum injected	—	—	2	104.9	D	6

IV	M4	Asibi	Normal <i>Aotus</i> (Control)	—	2.4	—	—	6	105.5	R	—
	M3	"	Normal <i>Alouatta</i> (Control)	—	3	—	—	7	105.9	R	—
	M1	"	Normal <i>Cebus albifrons</i>	—	3	—	—	2	105.9	D	5
	M2	"	(Control)	Asibi	3	14	No fever	—	103.8	R	—
	L19	"	<i>Ateles ater</i> 4 <i>Cebus albifrons</i> 3	"	3	31	Chronic fever	2	104.7	R	—
	M6	"	<i>Aotus</i> 2****	"	2.4	16	No fever	15	105.2	S	17
V	M7	"	<i>Cebus albifrons</i> 1****	"	2.2	14	No fever	—	103.7	R	—
	M8	"	<i>Alouatta</i> 2****	S.R.	3	18	13	—	103.5	R	—
	M9	"	<i>Saimiri</i> 23	"	1.9	38	32	—	103.6	R	—
	M10	"	Normal <i>Ateles ater</i> (Control)	—	2.8	—	—	2	105.4	D	6

\* Blood taken before second inoculation of virus (immunity test).

\*\* Blood taken 39 days after original inoculation and 14 days after immunity test.

\*\*\* Experiment includes repetition of tests on *Lagothrix* Nos. 2 and 3, using larger amounts of serum than in Experiment I.

\*\*\*\* Serum was taken from these animals, before they were given virus, for use in Experiment IV.

D = death. R = recovered. S = sacrificed; animal had tuberculosis.

was sometimes noted in the tarsal plates and in the intima of the aorta. Microscopically, necrosis and a great disorganization of architecture were often found in the liver. The hepatic lesions had not the typical and clear-cut distribution noted in *Macacus rhesus*, but apparently affected primarily the mid-zone. There was a great invasion of leucocytes, of both mononuclear and polymorphonuclear types. Nuclei of degenerated cells were swollen and the space between nucleolus and cell membrane was usually almost lacking in chromatin. Although the routine fixation was in formaldehyde solution, hematoxylin and eosin staining sometimes showed intranuclear eosinophilic bodies which probably represented the inclusions described by Torres (3). There were noted at times a severe congestion of the vessels and occasional small hemorrhages. The kidneys showed varying degrees of parenchymatous degeneration, with cast formation; congestion was frequently present.

#### DISCUSSION

It has been possible to pass yellow fever virus from *M. rhesus* to the three species of Brazilian monkeys considered in this report and back to *M. rhesus*. The "woolly monkey," *Lagothrix lagotricha*, has proved relatively refractory to virus inoculations. Two "spider monkeys," *Ateles ater*, have been infected, but the lesions produced have not been typical of yellow fever. About 50 per cent of the "squirrel monkeys," *Saimiri sciureus*, used in experimentation undoubtedly have become infected and many have died; lesions at autopsy have been very suggestive of yellow fever.

At the present time there is very little in the literature relative to infection experiments with the species used by us. Aragão has mentioned in one of his papers (4) that Dr. José Teixeira had secured infections of *Saimiri* monkeys in the Instituto Oswaldo Cruz in Rio de Janeiro. In the discussion of Hindle's paper before the Royal Society of Tropical Medicine and Hygiene, Sir James Kingston Fowler (5) said that as early as 1914 the West African Yellow Fever Commission had considered the use of New World monkeys in the study of yellow fever.

In Table III are noted the protection tests of sera from certain species not considered in the text. Some of these will be taken up in future publications. The "caiarára" monkey, *Cebus albifrons*, was discussed in a previous paper (2). At that time we had no data on the protective properties of the normal serum from this species. The serum of *C. albifrons* 4 was tested before the inoculation of virus (Table III, Experiment IV) and again 14 days after inoculation. The animal became immunized even though no fever developed. Six days after

inoculation, when the monkey had a temperature of 103.5°, blood was taken for transfer to *M. rhesus* J17. The latter did not react.

As noted in the protocols, two *Saimiri* monkeys which were known to have been infected (Nos. 13 and 23), were given a second inoculation of virus. In each instance the temperature subsequently rose to 104°, or higher, but apparently no virus reached the blood stream, because transfers of blood to *M. rhesus* produced no infections.

#### SUMMARY

1. *Saimiri sciureus* has been infected with yellow fever virus, both by the inoculation of infectious blood and by the bites of infective mosquitoes. Some of the monkeys have died, showing lesions, including hepatic necrosis, suggesting yellow fever as seen in human beings and in *rhesus* monkeys. Virus has been transferred back to *M. rhesus* from infected *Saimiri* both by blood inoculation and by mosquito bites. The virus undoubtedly has been maintained through four direct passages in *Saimiri*. Reinoculations of infectious material into recovered monkeys have not given rise to invasion of the blood stream by virus. Sera from recovered animals have protected *M. rhesus* against the inoculation of virus.

2. It has been possible to pass the virus to and from *Ateles ater* by the injection of blood or liver and by the bites of mosquitoes. The livers from two infected animals have shown no necrosis. The serum from one recovered monkey proved to be protective for *M. rhesus*.

3. Only three out of twelve *Lagothrix lagotricha* have reacted to yellow fever virus by a rise in temperature. Probably none have died as a result of the infection. In only one instance has the virus been transferred back to *M. rhesus*. The sera of recovered animals have had a protective action against yellow fever virus.

The writer gratefully acknowledges the help of his fellow workers, Dr. J. A. Kerr and Mr. Raymond C. Shannon, in some of the experiments here reported.

The identification of the "squirrel monkey," *Saimiri sciureus*, was given by Mr. Tate, of the American Museum of Natural History, in a personal communication to Dr. Sawyer.

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## EXPLANATION OF PLATE 12

FIGS. 1 and 2. Liver tissue of *Saimiri* monkey No. 3, which died following inoculation with yellow fever virus of the Asibi strain. Hematoxylin and eosin. Fig. 1  $\times 145$ . Fig. 2  $\times 462$ .



FIG. 1

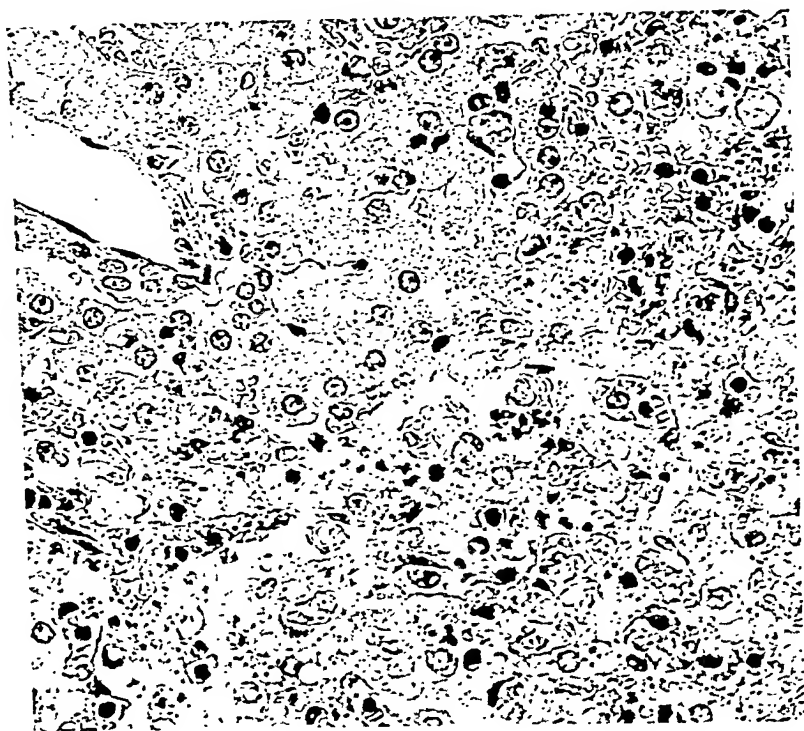


FIG. 2

(Davis: The transmission of yellow fever)





# THE EFFECT OF THE ROUTE OF IMMUNIZATION ON THE IMMUNITY RESPONSE TO PNEUMOCOCCUS TYPE I

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(Received for publication, March 1, 1930)

It has been shown that, following repeated inhalations of living Type I pneumococci, agglutinins and protective antibodies are demonstrable in the serum of rabbits (1). By the spray method employed, however, the number of bacteria lodging within the respiratory tract or invading the tissues cannot be controlled. The following study was undertaken in order to determine the immunity response of rabbits injected with fixed amounts of heat-killed pneumococci following (1) intravenous, (2) intraperitoneal, (3) intramuscular, and (4) subcutaneous injection of varying amounts of suspension of heat-killed pneumococcus Type I. The duration of active immunity and the length of time that agglutinins and protective antibodies persist in the serum will be dealt with in a subsequent paper.

## *Method*

The inoculum was composed of washed heat-killed Type I pneumococci suspended in salt solution. 1 cc. was equivalent in bacterial content to 10 cc. of an 18 hour broth culture. The rabbits were injected at 4 day intervals. The first 2 doses given were 0.5 cc., the next 2 doses 1 cc. each, and thereafter 1.5 cc. Different groups of rabbits received a total of 1, 3, 6, 9, 12 and 15 cc. of the inoculum. 10 days after receiving the last dose the animals were bled from the ear vein.

The presence of agglutinins was determined by a modified thread reaction. To 1 cc. of immune rabbit serum diluted in salt solution was added 0.2 cc. of an actively growing broth culture of pneumococcus Type I. The tubes were incubated for 2 hours in the water bath at 37°C., placed in the ice box overnight, and the reactions read the next morning. Agglutinins were recorded as present in the serum only when the reactions were positive in a dilution of at least 1:10.

The presence of protective antibodies in the blood of the immunized rabbits was demonstrated by determining the capacity of a given amount of serum, to protect white mice against intraperitoneal injection of homologous pneumococcus, the virulence of which was such that 0.000,001 cc. killed control mice within 48 hours. The immune serum and culture were administered simultaneously.

TABLE I

Agglutinins and protective antibodies in rabbits inoculated by

No. of inoculations	Total amount of inoculum	Intraperitoneal									Intramuscular			Subcutaneous		
		Intravenous			Intraperitoneal			Intramuscular			Intramuscular			Subcutaneous		
		Agglutini- mins	Amount of culture against which 0.5 cc. of sera protected	Aggluti- mins	Amount of culture against which 0.5 cc. of sera protected	Aggluti- mins	Amount of culture against which 0.5 cc. of sera protected	Aggluti- mins	Amount of culture against which 0.5 cc. of sera protected	Aggluti- mins	Amount of culture against which 0.5 cc. of sera protected	Aggluti- mins	Amount of culture against which 0.5 cc. of sera protected	Aggluti- mins	Amount of culture against which 0.5 cc. of sera protected	Aggluti- mins
2	1 cc.	A 1	.01	B 1	.001	C 1	.01	D 1	.1					D 1	.1	
		A 2	.01	B 2	.001	C 2	.001	D 2	.001					D 2	—	
		A 3	.01	B 3	.01	C 3	.01	D 3	.01					D 3	—	
4	3 cc.	A 4	1.100	B 4	.1	C 4	.1	D 4	.1					D 4	.000,1	
		A 5	1.50	B 5	.001	C 5	.001	D 5	—					D 5	.01	
		A 6	1.40	B 6	.1	C 6	.1	D 6	—					D 6	.01	
6	6 cc.	A 7	1.100	B 7	.001	C 7	.001	D 7	.001					D 7	.001	
		A 8	—	B 8	.01	C 8	.01	D 8	.1					D 8	.001	
		A 9	1.50	B 9	.1	C 9	.1	D 9	—					D 9	.000,01	
8	9 cc.	A 10	1.40	B 10	.1	C 10	.01	D 10	.000,01					D 10	.000,01	
		A 11	1.50	B 11	.01	C 11	.01	D 11	.001					D 11	.000,01	
		A 12	1.100	B 12	.1	C 12	.1	D 12	.000,01					D 12	.000,1	
10	12 cc.	A 13	1.500	B 13	.1	C 13	.1	D 13	.000,1					D 13	.000,01	
		A 14	1.100	B 14	.1	C 14	.1	D 14	.001					D 14	.1	
		A 15	1.500	B 15	.1	C 15	.1	D 15	.01					D 15	.1	
12	15 cc.	A 16	1.100	B 16	.1	C 16	.1	D 16	.01					D 16	.000,1	
		A 17	1.500	B 17	.1	C 17	.1	D 17	.001					D 17	—	
		A 18	1.500	B 18	.01	C 18	.01	D 18	.001					D 18	.001	
		A 19	1.400	B 19	.01	C 19	.01	D 19	.001					D 19	—	
		A 20	1.200	B 20	.1	C 20	.01	D 20	.000,1					D 20	—	
						C 21	.01	D 21	.000,1					D 21	—	

Before inoculation a test bleeding was taken from all the rabbits used. None of the normal rabbit sera contained agglutinins nor did the normal sera protect mice against intraperitoneal injections of 0.000,001 cc. of pneumococcus.

### *Agglutinins*

From Table I it is seen that 16 of the 20 rabbits which received, during the course of treatment, from 1 to 15 cc. of killed culture intravenously, developed agglutinins, as tested by the method employed, ranging in titre from 1-40 to 1-500. 3 of the 4 animals in whose serum agglutinins were not demonstrable had received only 2 doses totaling 1 cc. of original culture. Within the limits defined, the agglutinin titre of the immune sera rose as a rule in direct proportion to the amount and number of inoculi which the rabbits had received.

Of the 20 rabbits injected intraperitoneally, 12 developed demonstrable agglutinins. Although 1 of the rabbits which had received a total of 1 cc. developed agglutinins, the percentage was not notably increased among the rabbits that had received 3 and 6 cc. and 1 rabbit failed to show agglutinins even after the administration of a total of 15 cc. The titre of the agglutinins was also lower, being as a rule no higher than 1-50. Only 2 rabbits developed really active agglutinating sera, of 1-100 and 1-500 respectively.

In the case of the rabbits injected intramuscularly the percentage of those showing agglutinins in the blood is less. Of 21 rabbits treated intramuscularly only 7 showed serum agglutinins. In these instances, the agglutinin titre bore little relationship to the amount of vaccine injected. Although the sera of 2 rabbits which had received in all 3 and 6 cc. respectively showed agglutinins, only 1 of the 4 rabbits which had received a total of 15 cc. produced agglutinins. In all instances the agglutinin titre remained low. In only one instance were agglutinins demonstrable in a dilution as high as 1-20.

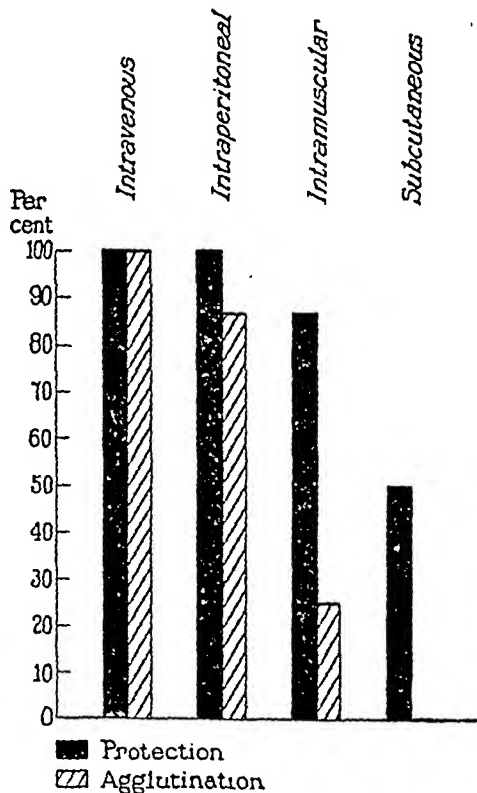
In the case of the 21 rabbits vaccinated subcutaneously none formed demonstrable agglutinins.

### *Protective Antibodies*

From Table I it is seen that all of the 20 rabbits inoculated intravenously developed protective antibodies. The serum of the 3 rabbits which received a total of 1 cc. protected mice against 0.01 cc.

of virulent Type I pneumococci while the sera of the remaining rabbits protected mice against 0.1 cc. of this culture.

The serum of the intraperitoneally injected rabbits also afforded protection in all instances. The amount of protection, however, in most instances was not so great as that exhibited by the serum of the



TEXT-FIG. 1. Comparison of immunity response of rabbits inoculated by various routes with 12 or 15 cc. of heat-killed pneumococcus vaccine

intravenously treated rabbits. In only 9 instances did the rabbit serum protect mice against 0.1 cc. of virulent culture.

The serum of 80 per cent of the intramuscularly injected rabbits afforded some degree of passive protection, but the protective power of the serum was in general less than that of the rabbits inoculated by the intravenous or intraperitoneal route. There was even more irregularity in the amount of protection afforded by the serum from the rabbits which had received the larger amounts of killed culture. The

serum of 2 rabbits which had received respectively a total of 3 and 6 cc. protected against 0.1 cc. of culture. Of the 4 rabbits which had received 15 cc., 1 failed to show any protective antibodies, and 3 protected mice only against 0.001 cc. of pneumococcus culture.

The differences just noted were even more conspicuous in the case of rabbits injected subcutaneously. Although the serum of 71 per cent of these rabbits showed some degree of protection, in most instances this was merely sufficient to protect mice against 0.001 cc. of culture.

The differences in the antibody response of rabbits inoculated by various routes is graphically shown in Text-fig. 1. 12 cc. of the saline suspension of heat-killed pneumococci, Type I, administered to rabbits intravenously proved to be a sufficient amount to insure the development of a high proportion of agglutinins and protective antibodies. In Text-fig. 1 the percentage of rabbits that had received 12 or 15 cc. of inoculum by various routes is graphically shown. The rabbits injected intravenously or intraperitoneally all developed protective antibodies in their serum. Even the rabbits immunized by intramuscular and subcutaneous injection developed protective bodies in most instances. The differences in formation of agglutinins in these same rabbits were striking. Whereas 100 per cent of the intravenously immunized rabbits showed agglutinins, only 87 per cent of the intraperitoneally and 25 per cent of the intramuscularly immunized rabbits produced agglutinins. Those immunized subcutaneously showed none.

#### *Relation between Agglutinins and Protective Antibodies*

From Table I it is also seen that 35 or 42 per cent of the 82 rabbits injected in various ways developed both agglutinins and protective antibodies. The serum of 37 animals showed only protective antibodies; while in 10 instances there was no demonstrable antibody response. In no instance were agglutinins present without protective antibodies. If the serum contained agglutinins, it would also, as a rule, protect mice against 0.1 cc. of culture. This occurred in 26 instances. But in 9 instances a serum containing agglutinins only protected mice against from 0.01 cc. to 0.000,01 cc. of pneumococcus culture. On the other hand, 5 sera which contained no demonstrable agglutinins protected mice against 0.1 cc.; and 12 sera protected against 0.01 cc. of virulent culture.

## DISCUSSION

Since the first demonstration that the serum of animals injected with pneumococci possessed immune properties a great deal of work has been done on the production of pneumococcus immunity. This work has recently been reviewed by Barach (2). Comparatively little attention, however, has been paid to different routes of inoculation. Cecil and Stephen (3) found that intravenous inoculation of small quantities of pneumococcus Type I completely protected monkeys against infection by the homologous organism whereas larger intramuscular doses failed. They also found that intratracheal inoculation would produce immunity but that spraying the throat did not produce complete immunity. Cooper (4) found that submucous inoculation of rabbits in the cheek afforded protection but that a similar inoculum subcutaneously or intradermally in the abdominal wall did not protect rabbits.

Lister (5) showed that the serum of rabbits inoculated intravenously contained agglutinins and opsonins but that the sera from other rabbits inoculated with similar amounts subcutaneously contained no agglutinins and that the opsonic index was only slightly raised.

The present experiments show that while agglutinins and protective antibodies are often both present in an immune serum for pneumococcus Type I their amounts do not necessarily vary together nor are both always found. Intravenous inoculation of rabbits will most regularly give rise to sera containing the two in quantity. Intraperitoneal and intramuscular inoculation elicit them to a less degree. But, following subcutaneous inoculation, according to the method here described, rabbits develop protective antibodies only.

## SUMMARY

1. The sera of 80 per cent of the rabbits intravenously inoculated with fixed amounts of heat-killed pneumococci contained agglutinins and all showed protective antibodies.
2. The sera of 60 per cent of the intraperitoneally inoculated rabbits contained agglutinins and all showed protective antibodies.
3. The sera of 33 per cent of the intramuscularly inoculated rabbits contained agglutinins and 86 per cent also showed protective antibodies.

4. None of the sera of the subcutaneously inoculated rabbits contained agglutinins although protective antibodies were present in 71 per cent.

5. Although there is a close relationship between the presence of agglutinins and protective antibodies in a given immune serum, these do not run parallel.

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# EXPERIMENTAL EPIDEMIOLOGY OF TUBERCULOSIS

## THE EFFECT OF CROWDING UPON TUBERCULOSIS IN GUINEA PIGS, ACQUIRED BY CONTACT AND BY INOCULATION

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In conjunction with the extensive studies at The Henry Phipps Institute on the contagion of tuberculosis of man (1) Perla (2) has investigated the experimental epidemiology of tuberculosis in guinea pigs. He found that normal guinea pigs confined with tuberculous cage mates acquired tuberculosis of alimentary origin, characterized by a marked involvement of the mesenteric and cervical lymph nodes. Guinea pigs confined in the same room with tuberculous animals but not in the same cage acquired a disease of respiratory type, characterized by extensive lesions of the lungs and of the tracheobronchial lymph nodes. The incidence of the disease increased with the intensity and duration of the exposure. He has also shown (3) that guinea pigs inoculated intraperitoneally with tubercle bacilli, such as those used as sources of contagion in these experiments, often excrete virulent microorganisms in the faeces and urine during the first week after inoculation, and always in the last stages of the disease. His work does not, however, show conclusively the effect of crowding upon the incidence of "contact" tuberculosis, because the intensity of exposure in his experiments was not the same in each degree of crowding. By confining a variable number of guinea pigs with a fixed number of tuberculous animals in cages of the same size, the approximately equal amounts of tubercle bacilli available were distributed in "doses" of different size, according to the number of animals exposed. At the suggestion of Dr. Eugene L. Opie, these studies have been continued by the writer.

In judging these experiments it must be borne in mind that the experimental epidemiology of tuberculosis entails greater difficulties

than a similar study of a more acutely fatal disease. The laborious nature of the experiments, extending over long periods of time, the chronicity of the disease, the relatively low incidence of tuberculosis acquired naturally by guinea pigs precludes the rapid demonstration of facts.

The results of Bruno Lange (4) of the Koch Institute on feeding guinea pigs with tubercle bacilli illustrate the difficulty last mentioned. He placed one or two drops of finely suspended, highly virulent tubercle bacilli in the mouths of guinea pigs by means of a pipette and found that although they can occasionally be infected by this method, even with quantities as low as 0.000,001 mg., they do not invariably develop tuberculosis even when 0.1 mg. is given. The disease so produced resembles the naturally acquired cage infection and is characterized by extensive lesions of the cervical and mesenteric lymph nodes without any evidence of tuberculosis at the portal of entry.

### *The Effect of Crowding upon the Incidence of Contact Tuberculosis*

*Method.*—72 guinea pigs were divided into 3 lots of 24 each. The first group was separated into 4 cages of 6 each; the second, into 6 cages of 4 each; and the third, into 12 cages of 2 each. Half of the guinea pigs in each cage were inoculated intraperitoneally with 0.000,01 mg. of a virulent human strain of tubercle bacilli, P-489A, to serve as sources of contagion for the other half, the normal animals, or "contacts." At the same time 12 normal animals without tuberculous cage mates, were placed into 4 cages, 3 per cage, as controls, to act as an index of the air-borne tuberculous contagion of the room.

All the guinea pigs used as contacts and as controls were obtained from The Rockefeller Institute at Princeton. The cages were all made of metal, with wire-mesh doors, 14 inches in height and width and 15 inches in depth. Upon the floor of each cage there was a rectangular pan 12 by 14 inches. To this uniform area of 168 square inches were confined the 6, 4 and 2 guinea pigs representing the three degrees of crowding. Upon the floor food was placed daily, consisting of hay, oats and bread soaked in water, with fresh, green vegetables twice a week, in amounts corresponding to the number of animals in each cage. The cages were cleaned 3 times weekly on alternate days. In case of the death of any animal from whatever cause its cage was sterilized.

We thus could study the contagion of tuberculosis under conditions that gave different degrees of crowding but presumably the same intensity of exposure. For each of the 12 contacts in the three degrees of crowding there was one inoculated animal in the cage to serve as its

source of contagion, the only variable being the whole number of animals confined to the same space. The population of the cages was maintained constant throughout the period of over a year by replacing the dying animals, either the inoculated or the contacts, by similarly infected or normal animals.

Toward the end of the experimental period all the surviving contacts were given two successive tuberculin tests intracutaneously at an interval of 2 months. If even the slightest positive reaction appeared, the animal was killed and carefully autopsied, and if there was the least doubt as to the presence of tuberculosis, virulent or living microorganisms were sought by means of culture and animal inoculation.

*Results.*—None of the 12 controls used as index of the air-borne contagion of the room developed tuberculosis.

In the 4 most crowded cages, in each of which there were 6 guinea pigs, 3 infected and 3 normal, 4 deaths with tuberculosis occurred. The data are summarized in Table I.

In the protocols of this and subsequent papers a disease characterized by an extensive tuberculosis of the mesenteric with little or no affection of the tracheobronchial nodes is considered of enteric origin and a massive affection of the tracheobronchial nodes with slight or no involvement of the mesenteric is interpreted as a respiratory infection. The experimental basis for this conclusion is the subject of the last paper of this series.

In Cage 1, there were two deaths with tuberculosis. Guinea pig 2 died at the end of 93 days of exposure. One mesenteric node measures 16 x 10 mm. and is caseated in the center; there are two smaller caseous nodes in the mesentery. There are a few isolated characteristic tuberculous foci in the lung, liver and spleen. The tracheobronchial and periportal nodes are somewhat enlarged, fibrous and caseous. Acid-fast bacilli were demonstrated in smears from the lung. Both lungs show severe congestion, which was probably the cause of death. The infection is chiefly of enteric origin.

Guinea pig 4 died at the end of 199 days of exposure. The mesenteric nodes form a chain of extremely hardened and enlarged masses, measuring 40 x 15 mm., with isolated foci of caseation. The tracheobronchial lymph nodes are much less enlarged and fibrous. The periportal nodes present an extensive tuberculosis with fibrosis and caseation. There is a massive fibrous nodular tuberculosis of the lungs, a moderate tuberculosis of the spleen and healing in the liver. Acid-fast bacilli were demonstrated in the lung. The route of infection here is to a great extent enteric.

The remaining contacts in this cage, exposed from 213 to 370 days, failed to develop tuberculosis.

In Cage 2, one death from tuberculosis occurred. Guinea pig 6 died at the end of 270 days of exposure. The mesenteric nodes are caseous. One measures 26 x 16 mm.; another about 10 mm. There is an extensive generalized tuber-

TABLE I

*Incidence of Contact Tuberculosis in Cages Containing Three Normal and Three Tuberculous Guinea Pigs Each*

Cage number	Total number of inoculated animals in cage during experiment	Number of the contact guinea pig	Days of exposure	Presence of tuberculosis in the contacts
1	10	1	370	none*
		2 D	93	+
		3	271	none*
		4 D	199	+
		5	213	none*
2	13	6 D	270	+
		7	134	none*
		8 K	165	none
		9	394	none*
3	18	10 D	133	+
		11	271	none*
		12	372	none*
		13 K	369	none**
4	16	14 K	359	none***
		15 K	365	none***
		16 K	365	none***

\* Absence of tuberculosis determined by repeated tuberculin tests.

\*\* Absence of tuberculosis confirmed by animal inoculation.

\*\*\* Absence of tuberculosis confirmed by animal inoculation and culture.

D = died; K = killed.

culosis of the lungs, liver, spleen and lymphatic systems. The infection in this animal is probably of enteric origin, largely.

The remaining three contacts, exposed for 134, 165 and 394 days, showed no evidence of tuberculosis.

In Cage 3, one animal, Guinea pig 10, died of tuberculosis at the end of 133 days of exposure. There are 10 mesenteric nodes varying in size from 15 to 5 mm.; all are fibrous and caseous. There is an extensive generalized tuberculosis of the

liver, spleen, lungs and lymphatic system. The infection is largely enteric in origin. A pulmonary nodule revealed acid-fast bacilli.

The remaining contacts, exposed for 271, 369, and 372 days, failed to show any tuberculosis.

In Cage 4, no case of tuberculosis developed amongst the contacts although all three animals had survived the entire experimental period. Tuberculosis could not be demonstrated in them either by autopsy, animal inoculation, or culture.

From Table I it will be seen that there was no direct correlation between the incidence of tuberculosis amongst contacts and the total number of tuberculous animals to which these were exposed. Thus in Cage 1, where there were about 3 replacements of the sources of contagion, totalling 10 tuberculous animals, 2 of the contacts developed tuberculosis, whereas in Cage 4, where there were 5 replacements, the same 3 guinea pigs successively exposed to 16 tuberculous cage mates failed to show any tuberculosis. Moreover it is seen that although the average duration of exposure of the contacts that died from tuberculosis was 173 days, 7 out of the 12 original contacts survived the entire experimental period of 359 to 384 days without developing tuberculosis.

In the second degree of crowding, where there were 4 guinea pigs per cage, 2 contacts developed tuberculosis. The data are summarized in Table II.

Guinea pig 3, in Cage 1, died at the end of 143 days of exposure. The chief node at the root of the mesentery measures 15 x 10 mm. and shows a caseous focus, and another mesenteric node shows a similar condition. The tracheobronchial and periportal nodes are moderately enlarged and caseous. There are isolated tuberculous foci in the liver and spleen. Acid-fast bacilli were demonstrated in the mesenteric and tracheobronchial nodes and in the spleen. The route of infection is probably both alimentary and respiratory. The cause of death was enterocolitis.

Guinea pig 8, in Cage 3, died at the end of 179 days of exposure. The deep cervical nodes on both sides measure 13 x 8 mm. and are fibrous and caseous. The mesenteric nodes are massive, measuring 33 x 20 mm., and contain caseous foci. There are circular band-like ulcers in the ileum. There is a generalized tuberculosis of the spleen, liver, lungs, and lymphatic system. This is an infection of enteric origin.

All the remaining contacts, exposed for intervals of 132 to 403 days, failed to show any evidence of tuberculosis although 5 of the 12 origi-

nal contacts survived the entire experimental period. Again no correlation could be noted between the incidence of the naturally acquired disease and the total number of tuberculous animals to which the contacts were exposed.

TABLE II

*Incidence of Contact Tuberculosis in Cages Containing Two Tuberculous Guinea Pigs Each*

Cage number	Total number of inoculated animals in cage during experiment	Number of the contact guinea pig	Days of exposure	Presence of tuberculosis in the contacts
1	10	1 D	275	none
		2	132	none*
		3 D	143	+
		4	262	none*
2	7	5	391	none*
		6 K	369	none**
3	8	7	403	none*
		8 D	179	+
		9	240	none*
4	10	10 D	269	none
		11	142	none*
		12 K	371	none**
5	11	13 K	366	none***
		14 D	89	none
		15	346	none*
6	10	16 K	366	none***
		17 D	267	none
		18	170	none*

\* Absence of tuberculosis determined by repeated tuberculin tests.

\*\* Absence of tuberculosis confirmed by animal inoculation.

\*\*\* Absence of tuberculosis confirmed by animal inoculation and culture.

D = died; K = killed.

Not one case of contact tuberculosis occurred under the least crowded conditions, in the 12 cages in each of which one normal animal was confined with one tuberculous animal, although 8 of the exposed

guinea pigs survived over a year. These data are summarized in Table III.

TABLE III

*Incidence of Contact Tuberculosis in Cages Containing One Normal and One Tuberculous Guinea Pig Each*

Cage number	Total number of inoculated animals in cage during experiment	Number of the contact guinea pig	Days of exposure	Presence of tuberculosis in the contacts
1	3	1 D	271	none
		2	165	none*
2	5	3 D	207	none
		4 D	95	none
		5	115	none*
3	3	6 D	165	none
		7	262	none*
4	5	8 D	186	none
		9	224	none*
5	2	10	421	none*
6	2	11	379	none*
7	4	12	421	none*
8	4	13	385	none*
9	3	14	399	none*
10	3	15	314	none*
11	5	16	421	none*
12	3	17	421	none*

\* Absence of tuberculosis determined by repeated tuberculin tests.

D = died.

*Summary and Discussion.*—During the entire year 4 instances of contact tuberculosis occurred in the most crowded cages, where 3 normal guinea pigs were confined with 3 tuberculous cage mates. Two



contacts developed tuberculosis in the second degree of crowding, where 2 normal guinea pigs were exposed to 2 tuberculous cage mates. No case of contact tuberculosis occurred in the least crowded cages, where one normal guinea pig was exposed to one tuberculous animal. Essentially similar results were obtained the following year with a larger series of animals, where crowding was one factor amongst others studied; this experiment will be reported in a subsequent paper.

Thus it appears that the incidence of contact tuberculosis is increased by crowding, when the intensity of exposure is the same in that one tuberculous cage mate serves as a source of contagion for each animal exposed. Nevertheless it is evident that where one normal guinea pig is confined with one tuberculous animal to a certain area, the normal animal is exposed to the excreta of that one guinea pig, but when 3 normal guinea pigs, for example, are confined with 3 tuberculous cage mates to the same area, each of these 3 is exposed to 3 inoculated animals. It was shown by Perla that tuberculous guinea pigs excrete tubercle bacilli only during the first week and at the end of the disease. It is apparent that the chances for contagion are much greater in the more crowded cages because the individual sources of contagion are in different stages of the disease at the same time, and tubercle bacilli are being excreted at more frequent intervals than in the less crowded cages.

Furthermore, as will be shown, the course of tuberculosis in the inoculated guinea pigs was also influenced by crowding, there being a tendency for a longer period of survival and a more chronic type of disease in the less crowded animals. This again will tend to diminish the intensity of exposure in the less crowded cages. Thus it appears that an important factor in crowding is the "dosage" of bacilli available. However, that there are numerous other factors in crowding beside dosage that influence contact tuberculosis is evidenced by the fact that there is no direct correlation between its incidence and the intensity and duration of exposure. The work of Lange (4) and others with feeding experiments points in the same direction.

Undoubtedly such variables as the native resistance of the individual guinea pig to tuberculosis (5), the ingestion and penetration of the organism, both as regards the quantity absorbed and the intervals between absorptions, and, possibly, the gradual immunization of some

animals by repeated "doses" of suitable sizes at suitable intervals, may influence the incidence and course of tuberculosis acquired by contagion.

In regard to the last possibility, it is noteworthy that of the 6 contacts that died of tuberculosis in these experiments, 5 died within the first 7 months of exposure. The sixth died 2 months later. None died thereafter, nor was any tuberculosis or tubercle bacilli found in the 20 of the original 36 contacts that survived over 1 year.

The character of the disease in the 6 guinea pigs that acquired it by contact has been a generalized progressive tuberculosis; in only one of them was there any evidence of healing in the liver. It has been marked by an extensive involvement of the mesenteric lymph nodes. The tracheobronchial lymph nodes have also been involved, but usually to a much smaller degree. Lange (4) has shown that tubercle bacilli could be isolated from the cervical and mesenteric lymph nodes and from the spleen of guinea pigs from 1 to 3 days after feeding them with the microorganism, but they were not found in the lungs and tracheobronchial lymph nodes until later. The early appearance of tubercle bacilli in the spleen accounts for the tuberculous affection of the lung, tracheobronchial lymph nodes and other organs, observed soon after the enteric infection. In the 6 guinea pigs reported here, the route of infection appears, therefore, to be largely enteric, although the generalized character of the disease often obscures it and simultaneous respiratory infection sometimes occurs, especially in the less crowded cages.

#### *The Effect of Crowding on the Course of Tuberculosis in Inoculated Guinea Pigs*

It was shown by Webster (6) that if mice are given a certain quantity of mouse typhoid bacilli *per os* and distributed in different degrees of crowding the mortality is higher in the more crowded cages.

A similar effect of crowding on the course of tuberculosis in guinea pigs inoculated intraperitoneally with tubercle bacilli was noted in these as well as in other experiments.

In Table IV are summarized the average duration of life and the incidence of chronic tuberculosis amongst 531 guinea pigs used in experiments described here

and to be reported later; they had been inoculated intraperitoneally with 0.001 and 0.000,01 mg. of the same strain of tubercle bacilli and distributed in groups of 1, 2 and 3 animals per cage, with an equal number of normal cage mates. Only those guinea pigs are included that died of tuberculosis. Two types of cage housed these animals: ordinary cages with a pan as floor, as described above, and special cages in which the faecal boluses fell through a wire-mesh floor into a pan 3 inches below. In the latter type, the food was placed in metal cups attached to the door and walls of the cage; in the ordinary cages the food was placed directly on the pan. For brevity's sake the individual data for each of these 531 guinea pigs are omitted and for that reason the probable error of the mean is included in the table

TABLE IV

*The Effect of Crowding on the Course of Tuberculosis in Guinea Pigs Inoculated Intraperitoneally with Tubercle Bacilli*

Dose.....	0.001 mg.								0.000,01 mg.			
Cage type...	Ordinary				Special				Ordinary			
Number of inoculated animals per cage	Total number of animals	Average duration of life	Probable error of mean	Percentage incidence of chronic tuberculosis	Total number of animals	Average duration of life	Probable error of mean	Percentage incidence of chronic tuberculosis	Total number of animals	Average duration of life	Probable error of mean	Percentage incidence of chronic tuberculosis
1									33	122	±5.8	54
2	64	125	±6.2	28	65	119	±7.3	30	42	107	±5.3	21
3	172	84	±3.0	17	73	100	±5.8	22	82	100	±3.7	18

Under the heading "chronic tuberculosis" are included those animals that showed various types of pulmonary affection, such as tuberculous bronchopneumonia, pulmonary tuberculosis with excavation or chronic fibrous pulmonary tuberculosis, with more or less healing in the liver, spleen and lymph nodes.

It is seen that there is a tendency to a higher incidence of chronic tuberculosis and a longer average duration of life amongst those guinea pigs that are less crowded. The 64 guinea pigs inoculated intraperitoneally with 0.001 mg. and distributed in ordinary cages in groups of 2 survived on an average of 41 days longer than the 172 guinea pigs similarly housed and inoculated with the same quantity and strain of bacilli by the same route but distributed in groups of 3 per cage. The incidence of chronic tuberculosis was 11 per cent higher in the less crowded cages.

The same general results were obtained in the special cages with the same dose and in the ordinary cages with 0.000,01 mg., when the three lots of inoculated guinea pigs in groups of 1, 2 and 3 animals per cage survived a successively shorter period with each increase in the number of tuberculous animals per cage and, inversely, the incidence of chronic tuberculosis increased with each decrease in the degree of crowding. Where there was 1 tuberculous animal per cage the inci-

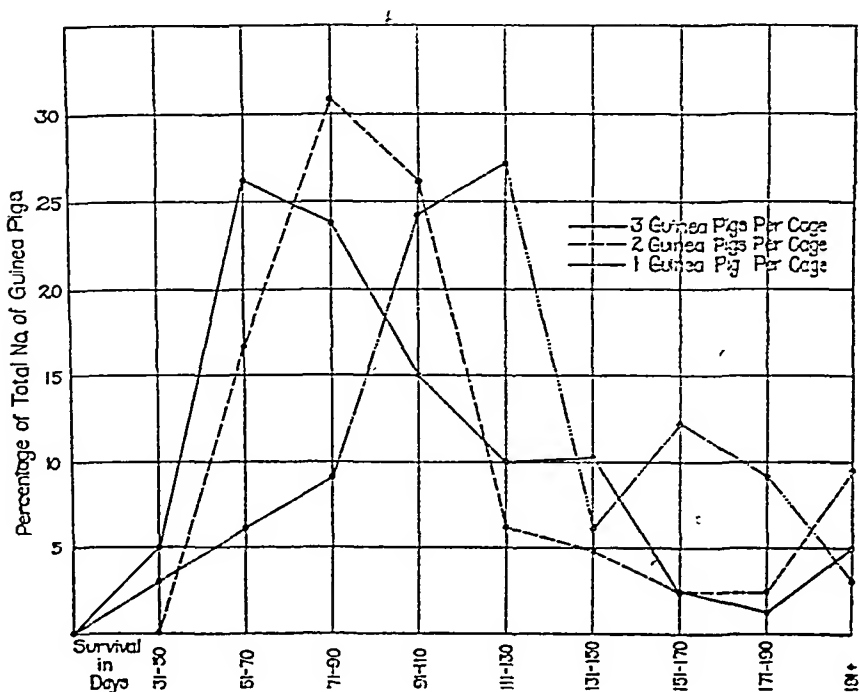


FIG. 1. Duration of survival in relation to degree of crowding

dence of chronic tuberculosis was 54 per cent, whereas where they were 3 per cage the incidence was 18 per cent. In the intermediate degree of crowding smaller differences were observed.

The percentage distribution of the duration of life in these three groups is illustrated in Fig. 1. It will be seen that with each decrease of crowding the mode moves to the right, or in other words that the interval of greatest frequency occurs at successively later times.

Where there were 3 tuberculous animals per cage 50 per cent died in from 51 to 90 days; 57 per cent of the guinea pigs in groups of 2 died in from 71 to 110 days and 51 per cent of those distributed 1 per cage survived 91 to 130 days. Thus, although some guinea pigs will succumb to tuberculosis early and others will resist the disease for a very long time irrespective of crowding—presumably because of the wide variations in individual resistance to tuberculosis—a large percentage are distinctly influenced by the degree of crowding in their length of survival and amongst these, the more crowded the guinea pigs, the shorter their duration of life from the same dose of tubercle bacilli.

Webster has shown that the higher mortality from typhoid fever amongst the more crowded mice was caused by superinfection. The obvious explanation for the tendency to a more acute type of tuberculosis and shorter life amongst the more crowded guinea pigs is constant exposure, after inoculation, to more tubercle bacilli. This is supported by the fact that where there were 3 tuberculous animals in ordinary cages the average duration of life of 172 guinea pigs was 84 days whereas of 73 guinea pigs similarly treated and similarly crowded in the special cages, in which the contact with excreta bearing tubercle bacilli was considerably reduced, the duration of life was 100 days. Furthermore the difference in the duration of life in the two degrees of crowding in the special cages is much less than that in the ordinary cages, a fact again attributable to the larger dosage available in the ordinary cages.

That other factors may be concerned is indicated by the fact that the life of 2 inoculated guinea pigs in the special cages is not longer than that of 2 inoculated guinea pigs in the ordinary cages. No pathological evidence of superinfection could be obtained as there was no greater tuberculous involvement of the mesenteric nodes of the more crowded inoculated animals.

#### CONCLUSIONS

1. If normal guinea pigs are confined with an equal number of tuberculous cage mates the incidence of "contact" tuberculosis is increased by crowding. This is probably due largely to an increase in the amount of tubercle bacilli available in the more crowded cages, although no constant relationship could be established between the

intensity of the exposure and the incidence of tuberculosis acquired by contagion. Other factors must be determined.

2. If guinea pigs are inoculated intraperitoneally with a given quantity of human tubercle bacilli and distributed in different degrees of crowding, the duration of survival is shortened in the more crowded animals, and the incidence of chronic types of tuberculosis is greater among the less crowded animals.

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# EXPERIMENTAL EPIDEMIOLOGY OF TUBERCULOSIS

## AIR-BORNE CONTAGION OF TUBERCULOSIS IN AN ANIMAL ROOM

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From the time of Koch (1) it has been known that normal guinea pigs confined in the same room, though not in the same cage, with tuberculous animals may acquire tuberculosis. Since then, many confirmatory reports have appeared, which have recently been reviewed by Perla (2). He also observed instances of tuberculosis so acquired and concluded that the route of infection in such cases is the respiratory tract. Many investigators, however, are still sceptical of the occurrence of tuberculosis under these conditions (3). Lydia Rabinowitsch-Kempner has recently published a questionnaire (4) on the subject. Amongst those who replied, Theobald Smith, Calmette and Uhlenhuth had never seen spontaneous tuberculosis, and Krause, Bruno Lange and Griffith had met with it, though extremely rarely. She concludes (5) from this as well as from her own work that although tuberculosis may be acquired naturally by rabbits and guinea pigs, the latter acquire tuberculosis but rarely when living together with tuberculous animals. Some writers (6) stress the hygienic conditions of the laboratory and the nutrition of the animals as determining factors. The following experiment was undertaken to obtain further information.

The main animal room of The Henry Phipps Institute, which measures 29 feet in length, 25 feet in width, and 16 feet in height—a space of 11,600 cubic feet—is accommodated with up to 270 metal cages, measuring 14 x 14 x 15 inches, with wire-mesh doors. These usually house about 2 animals per cage or a total of 540 animals. The cages are arranged on 4 metal stands of 3 tiers each. Upon each tier there are 2 rows of cages facing in opposite directions. Two normal guinea pigs, obtained from The Rockefeller Institute at Princeton, were placed in each of 27



of the cages and left undisturbed. These cages, painted black to make them conspicuous, were distributed evenly about the room and remained in the positions indicated in the figure (Fig. 1). The animals in all the other cages were variously treated during the different experiments in progress.

It is difficult to determine with exactitude what the actual, constantly changing tuberculous population in the animal room and its distribution in time and space has been throughout the period of these experiments—2 years and 8 months—

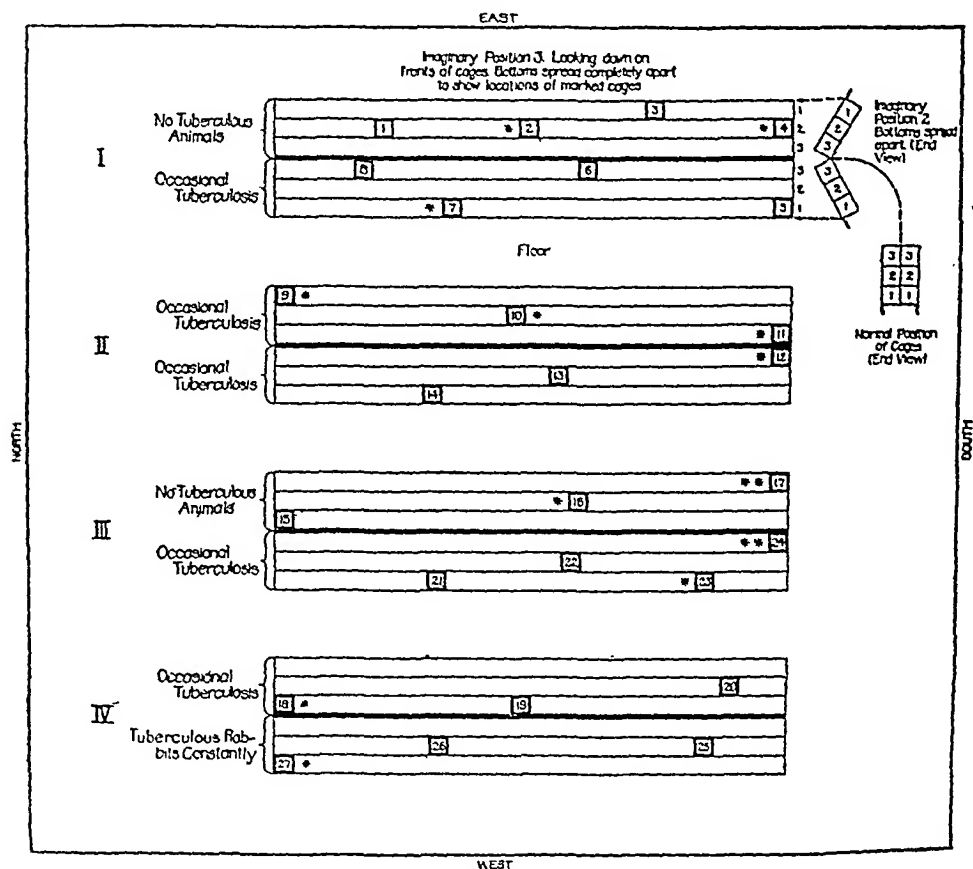


FIG. 1. Each star represents a case of contact tuberculosis

except that in the 3 tiers of cages adjoining and facing the west wall of the room there has been constantly a changing population of tuberculous rabbits. The 3 tiers of cages facing the east wall housed no tuberculous animals at any time; likewise the 3 tiers of Stand III facing the east; in the remaining cages there were often tuberculous animals.

The population of the 27 cages was maintained constant throughout this period by replacing the dying animals with normal guinea pigs. The general care and feeding of these animals has been described in a previous paper (7).

During the entire period 103 guinea pigs have been exposed in these cages. Of these, 58 died of other diseases some time during the 32 months; 15 died with tuberculosis; 21 were killed and showed no tuberculosis, and 9, which had survived the entire period and had shown repeatedly negative tuberculin reactions, were reserved for further experimentation.

The presence of tuberculosis was established on the basis of characteristic, unequivocal tuberculous lesions with characteristic acid-fast bacilli in the smears of these lesions, and, in the case of the slightest doubt, the conclusion was tested by animal inoculation.

In Table I are given the detailed observations in the 15 cases of tuberculosis. Eleven of these animals died of tuberculosis without any complicating cause. Their average survival was 551 days, ranging between 238 and 948 days of exposure. The disease was usually chronic in nature, characterized by a massive tuberculosis of the lungs, often (in 7 guinea pigs) with cavity formation. There was a massive fibrocaseous tuberculosis of the tracheobronchial lymph nodes with little or no involvement of the mesenteric and cervical nodes. Tuberculosis in the liver and spleen was usually only moderate in extent and often had healed in these organs, especially in the liver. In some cases there were lesions closely resembling human tuberculosis of the childhood type, one or several small nodules in the lung being accompanied by massive, caseated tracheobronchial lymph nodes. In others, however, pulmonary lesions occurred without any gross evidence of tuberculosis in the draining lymph nodes, or *vice versa*.

The experimental basis for the judgment as to the route of infection in these guinea pigs is presented in the last paper of this series.

The route of infection in the 3 guinea pigs in which the tuberculous lesions were limited to the tracheobronchial lymph nodes, the lungs or both, is obviously the respiratory tract. Five showed extensive pulmonary and tracheobronchial lesions as well as tuberculosis in other organs, but no affection of the mesenteric and cervical lymph nodes. The tuberculosis in these is therefore also definitely respiratory in origin. In 4 guinea pigs the route of infection is probably respiratory in the main, for although the mesenteric and cervical nodes are tuberculous the affection in the tracheobronchial lymph nodes is far greater. In Guinea pig 8 there was massive fibrous pul-

TABLE I

*Tuberculosis in Normal Guinea Pigs Exposed in a Room Where Tuberculous Animals Are Housed*

Cage No.	Guinea pig No.	Duration of exposure in days	Cause of death	Pathological observations	Route of infection
2	1	502	Tuberculosis	Extensive nodular pulmonary tbc.; extensive fibrocaseous tbc. of tracheobronchial lymph nodes; no tbc. of cervical and mesenteric lymph nodes; extensive tbc. of spleen and liver.	Respiratory tract
4	2	409	Tuberculosis	Extensive pulmonary tbc. with excavation; tracheobronchial lymph nodes slightly enlarged and caseous; single minute focus in mesenteric nodes; no tbc. in cervical lymph nodes; fibrosis of liver; no tbc. in spleen.	Respiratory tract
7	3	649	Tuberculosis	Extensive pulmonary tbc.; extensive fibrocaseous tbc. of tracheobronchial lymph nodes; no tbc. of cervical lymph nodes; slight tbc. of liver and spleen.	Respiratory tract ?
9	4	608	Tuberculosis and pleurisy	Moderate nodular pulmonary tbc. with excavation; extensive fibrocaseous tbc. of tracheobronchial lymph nodes; slight tbc. of mesenteric lymph nodes; tbc. of spleen; slight tbc. of liver with fibrosis.	Respiratory tract ?
10	5	624	Tuberculosis	Moderate pulmonary tbc.; extensive fibrocaseous tbc. of tracheobronchial lymph nodes; extensive tbc. of spleen and liver.	?†
11	6	538	Tuberculosis	Extensive pulmonary tbc.; extensive fibrocaseous tbc. of tracheobronchial lymph nodes; moderate tbc. of liver and spleen.	Respiratory tract ?
12	7	622	Tuberculosis	Slight pulmonary tbc.; all lymph nodes enlarged and caseous; extensive tbc. of spleen; moderate tbc. of liver.	?†
16	8	948	Tuberculosis	Massive fibrous pulmonary tbc. with excavation; tracheobronchial, mesenteric and cervical lymph nodes fibrous; fibrosis of liver; slight tbc. in enlarged spleen.	Respiratory tract ?

† These animals died in the summer in the absence of the writer and no note was made in regard to the involvement of the mesenteric nodes.

TABLE I—*Concluded*

Cage No.	Guinea pig No.	Duration of exposure in days	Cause of death	Pathological observations	Route of infection
17	9	615	Tuberculosis	Moderate fibrous pulmonary tbc. with excavation; massive fibrocaseous tbc. of tracheobronchial lymph nodes; no tbc. of mesenteric and cervical lymph nodes; slight tbc. with fibrosis in liver; moderate tbc. of spleen.	Respiratory tract
	10	513	Jaundice and tbc.*	Fibrous and fibrocaseous tbc. of tracheobronchial lymph nodes; no tbc. anywhere else in the body.	Respiratory tract
18	11	216	Tbc. and broncho-pneumonia	Moderate pulmonary tuberculosis; massive fibrocaseous tbc. of tracheobronchial lymph nodes; no tbc. in mesenteric; single focus in cervical lymph node; moderate tbc. of liver and spleen.	
23	12	238	Tuberculosis	Massive pulmonary tbc. with excavation; massive caseous encapsulated tracheobronchial lymph nodes; moderate caseous and fibrous tbc. of cervical, mesenteric and remaining lymph nodes; massive tbc. of liver and spleen.	Respiratory tract
24	13	257	Tuberculosis	Extensive pulmonary tbc. with excavation; massive fibrocaseous tbc. of tracheobronchial lymph nodes; no tbc. in cervical and mesenteric lymph nodes; no tbc. in liver; moderate tbc. of spleen.	Respiratory tract
	14	661	Tuberculosis	Chronic pulmonary tbc. with excavation; tracheobronchial lymph nodes enlarged and fibrous; no tbc. of mesenteric nodes; no tbc. in liver; moderate tbc. of spleen.	Respiratory tract
27	15	502	Pneumonia and tbc.*	Isolated pulmonary nodular tbc.; massive fibrocaseous tbc. of tracheobronchial lymph nodes; no evident tbc. anywhere else in the body.	Respiratory tract

\* Virulent tubercle bacilli demonstrated by animal inoculation in addition to characteristic acid-fast bacilli found in all of the 15 guinea pigs.

monary tuberculosis with excavations, healing in the liver and spleen and fibrosis of the lymphatic system. 'Here again the infection probably entered by way of the air passages. 'The remaining 2 guinea pigs died in the summer in the absence of the writer and no note was made in regard to the involvement of the mesenteric nodes; hence in these the route of infection could not be determined. Thus in most and perhaps all of the animals the route of infection was the respiratory tract.

Although the incidence of naturally acquired tuberculosis amongst the 103 guinea pigs is only 14.5 per cent, only 20 of the animals survived exposure for over 2 years. If the duration of exposure is considered in relation to the incidence of the disease, it becomes apparent

TABLE II

*Incidence of Tuberculosis in Normal Guinea Pigs Grouped According to Duration of Exposure in a Room Containing Tuberculous Animals*

	Duration of exposure				
	3 weeks to 6 months	6 months to 1 year	1 year to 1½ years	1½ years to 2 years	2 years to 32 months
No. exposed.....	15	33	18	17	20
No. developing tuberculosis.....	0	3	5	6	1
Percentage incidence of tuberculosis.....	0	9.1	27.7	35.3	5

that a much greater percentage would have died with tuberculosis had they all survived the entire experimental period.

In Table II it will be seen that none of the 15 guinea pigs exposed for a period of 3 weeks to 6 months developed tuberculosis. Of the 33 animals that were exposed from 6 months to 1 year 3 or 9.1 per cent developed tuberculosis. Two of these died of tuberculosis, one after 238 days and one after 257 days of exposure. The other died 216 days after the beginning of exposure with tuberculosis complicated by bronchopneumonia. Thus a considerable proportion of guinea pigs exposed to tuberculous animals under conditions that are common in many laboratories where investigations in tuberculosis are carried on, have developed an extensive tuberculosis after 7 or 8 months in such an environment.

Of 18 guinea pigs that survived an exposure of 1 to  $1\frac{1}{2}$  years, 5 or 27.7 per cent have developed tuberculosis. Of 17 guinea pigs that survived an exposure of  $1\frac{1}{2}$  to 2 years, 6 or 35.3 per cent developed tuberculosis. Thus the incidence of tuberculosis increases with the duration of exposure up to this point. But of 20 guinea pigs that have survived an exposure of 2 years to 2 years and 8 months, only 1 or 5 per cent developed tuberculosis. Thus it appears that after a certain length of time a further extension of exposure to the tubercle bacillus is followed by a decrease instead of a still greater increase in incidence of the disease. This striking fact will be considered presently.

The distribution of mortality and morbidity from tuberculosis has been more or less even over the entire room, as is indicated by the stars in Fig. 1. There has been no evidence that guinea pigs exposed in cages close to cages containing tuberculous animals have developed tuberculosis more often than those at a distance from them. Thus in the first 4 cages placed amongst animals none of which had been inoculated with tuberculosis, there were two cases of tuberculosis, whereas in Cages 25, 26 and 27, which were constantly surrounded by tuberculous rabbits, there was only one case of tuberculosis. Apparently the organism is more or less equally distributed over the entire room.

#### DISCUSSION

Under controlled conditions, then, guinea pigs confined in cages in a room where tuberculous animals are quartered have developed unquestioned tuberculosis, as seen at autopsy and confirmed by animal inoculation. In a subsequent paper the character of their disease will be compared with that acquired by guinea pigs living in the same cage with tuberculous animals. The incidence of the infection acquired by air-borne contagion was increased by the duration of the exposure up to a certain point. It rose from none in the first 6 months to 9.1 per cent in the first year, to 27.7 per cent in 18 months and to a maximum of 35.3 per cent in 2 years. Thereafter, however, only 1 out of 20, or 5 per cent, was affected. This marked decrease in incidence, following a still further increase in duration of exposure, can be explained by one or more of the following possibilities.

The 19 guinea pigs may be individuals that by some chance have escaped the effective disease-producing dosage of tubercle bacilli;

they may be individuals of such great innate resistance to tuberculosis that they are able to overcome quantities of bacilli that in other animals would cause disease; they may be those animals in which the equilibrium between contagion and resistance was such that by virtue of exposure to certain doses of bacilli suitably spaced in time an acquired resistance to the disease was produced.

That the first is not a likely explanation of these results is indicated by the fact that 6 guinea pigs exposed for some time during the same period in the same cages with these 19 guinea pigs have developed tuberculosis. Furthermore, the lungs and tracheobronchial lymph nodes of these resisting animals showed a moderate to an extensive anthracosis. It was shown by Augustine (8) that although the air of rooms where cases of open human pulmonary tuberculosis are confined rarely contained living tubercle bacilli, the dust of 25 per cent of such rooms contained virulent tubercle bacilli. She found no tubercle bacilli in samples of air in our animal room tested during the progress of these experiments. Unfortunately the dust was not examined, but in view of the comparatively high incidence of tuberculosis of air-borne character among animals confined in this room, it is reasonable to assume that here too, as in human dwellings, the organism is present in the dust. Yet in spite of the considerable penetration of this dust, laden, presumably, with tubercle bacilli, into the lungs and tracheobronchial lymph nodes of these 19 animals, as is shown by their anthracosis, they have failed to develop tuberculosis.

The other two possibilities seem to be more likely and work is now in progress with the aim of determining which of these factors, the innate or the acquired resistance, is responsible for the reduction in the incidence of tuberculosis in guinea pigs exposed for such long periods of time. It is for this purpose that the 9 animals referred to above, along with other animals derived from other sources, have been reserved.

The relatively high incidence of tuberculosis in guinea pigs confined in the same room with tuberculous animals for 7 or 8 months is pertinent to the present widespread discussion as to the existence of a filterable form of the tubercle bacillus. No inoculation chancre and no satellite lymph node involvement occur after the injection of filtrates of tuberculous material. However enlarged lymph nodes, usually tra-

cheobronchial nodes, containing acid-fast bacilli are found, and sometimes, though rarely, tuberculous lesions, usually limited to the lungs and tracheobronchial lymph nodes, have been seen (3, 9). It is evident that before a conclusion can be drawn from such cases the possibility of air-borne contagion must be ruled out.

#### CONCLUSIONS

1. Guinea pigs living in the same room but not in the same cage with tuberculous animals acquire tuberculosis, characterized by a chronic course, a marked involvement of the lungs, often with cavity formation and a massive tuberculosis of the tracheobronchial nodes; the mesenteric and cervical nodes are slightly or not at all affected.

2. The route of infection in these guinea pigs is almost always the respiratory tract.

3. Of 103 guinea pigs exposed for a period of up to 32 months 15 or 14.5 per cent developed tuberculosis. The shortest period of exposure leading to fatal tuberculosis was 8 months.

4. The incidence of this tuberculosis acquired by air-borne contagion increases with the duration and intensity of the exposure up to a certain point.

5. A large percentage of the guinea pigs weathered a continuous exposure to the tubercle bacillus for 32 months without becoming tuberculous. This may be due to an innate natural resistance against tuberculosis, or to an acquired immunity resulting from the continuous exposure to the contagion.

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# EXPERIMENTAL EPIDEMIOLOGY OF TUBERCULOSIS

## THE EFFECT OF ELIMINATING EXPOSURE TO ENTERIC INFECTION ON THE INCIDENCE AND COURSE OF TUBERCULOSIS ACQUIRED BY NORMAL GUINEA PIGS CONFINED WITH TUBERCULOUS CAGE MATES

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It was found by Perla (1) and confirmed in a previous paper (2) that guinea pigs exposed to tuberculous cage mates acquire a disease that is largely enteric in origin, the main source of contagion being the faecal matter of the inoculated animals. It was thought desirable to determine what effect if any would be exercised on the incidence and course of contact tuberculosis if normal guinea pigs are confined with tuberculous animals in a cage so constructed that the excreta do not remain in contact with the occupants or their food. Such a cage was devised.

It was made of metal, 14 inches in height and width and 15 inches in depth, with wire-mesh doors, like the cages used in the previous experiments except that, instead of a pan serving as a floor for the animals, the bottom of the cage was  $\frac{1}{4}$  inch wire-mesh through which the faecal boluses fell into a pan lying 3 inches below. The food was placed in metal cups attached to the door and walls of the cage.

### *Methods*

192 guinea pigs were separated into 4 groups of 48 each. Two groups were placed in the "special" cages described above. The other two groups were placed in ordinary cages like those used previously. In each cage type there were two degrees of crowding. One group of 48 guinea pigs was subdivided into 8 cages, 6 in each cage and a second group of 48 guinea pigs subdivided into 12 cages, 4 in each cage. Half of the animals in each cage were inoculated intraperitoneally with 0.001 mg. of the same strain of tubercle bacilli used in the earlier study (2),

and served as sources of contagion for the other half. There were thus 24 normal "contacts" in each of the 4 groups, distributed equally in two degrees of crowding in cages of two types. The population of all the cages was kept constant by replacing the dying animals, either the inoculated or the contacts, by similarly infected or normal animals. As with the previous study these experiments were continued for over a year.

Half of the contacts in each of the 4 groups were subjected to intracutaneous tuberculin tests beginning with the fourth month of the exposure and repeated at intervals of about 2 months thereafter, to determine if an animal once become sensitive to tuberculin will lose this sensitivity during the course of the exposure to the disease, and also to determine how long the disease lasted in those that eventually succumbed. The other half were left undisturbed. As controls for the air-borne tuberculous contagion of the room, 24 guinea pigs were subdivided into 6 cages, 4 plain and 2 special, each containing 4 animals and were left undisturbed during the course of the experimental period. The population of these cages was also maintained constant during this time.

Toward the end of the experiment all the surviving contacts and the controls for the room were tested twice with tuberculin at intervals of 1 month. If the result was positive the animal was isolated in a separate cage and observed until its death. Those that presented repeatedly negative tuberculin reactions were reserved for further study. Those that presented questionable reactions were killed and carefully autopsied. Animal inoculation and culture were also used in some cases to determine the presence of virulent or viable tubercle bacilli.

*Results.*—In the 8 ordinary cages, in each of which 3 normal guinea pigs were confined with 3 tuberculous animals, 7 developed tuberculosis. These data are summarized in Table I.

The experimental basis for judging the route of infection in these guinea pigs is given in a subsequent paper.

In Cage 2, Guinea pig 5 was exposed for the entire period of 370 days and died 449 days after the beginning of the experiment. The mesenteric lymph nodes are fibrocaseous and measure 25 x 15 mm. The mesenteric border of the intestines and the mesenteric vessels are studded with confluent nodular tubercles. The mucous membrane of the appendix is ulcerated. The tracheobronchial lymph nodes measure 15 x 12 mm. and are fibrous. There is a generalized tuberculosis of the lungs, liver and spleen. Acid-fast bacilli were demonstrated in the lung and in the mesenteric border of the intestines. The route of infection is undoubtedly enteric.

In Cage 4, Guinea pig 11 died on the 324th day of exposure. The nodes at the root of the mesentery form a huge matted fibrous mass with residual foci of caseation 40 x 26 mm. Many more mesenteric nodes are also extensively affected.

The cervical nodes, both the superficial and deep, show an extensive fibrocaseous tuberculosis. The tracheobronchial nodes measure  $20 \times 18$  mm. and are fibrous. There are discrete tubercles in the lung and spleen and a pneumonia of the right

TABLE I

*The Incidence of Contact Tuberculosis in Ordinary Cages Containing Three Normal and Three Tuberculous Guinea Pigs Each*

Cage No.	Total No. of inoculated animals in cage during experiment	Total No. of contacts in cage during experiment	The three contacts that survived the longest period	Duration of exposure	Presence of tuberculosis in the contacts	Days survived by contacts that developed tuberculosis
1	17	4	1 D	289	none	
			2	370	none*	
			3 D	361	none	
2	16	5	4 D	297	none	449
			5 D	370	+	
			6 D	292	none	
3	23	3	7	370	none*	
			8 D	326	none	
			9	370	none*	
4	17	7	10	215	none*	324
			11 D	324	+	
			12	370	none*	
5	16	3	13	370	none*	
			14	370	none*	
			15	370	none*	
6	21	4	16 D	370	+	467
			17 D	370	+	440
			18 D	266	+	379
7	25	4	19 D	303	+	303
			20	370	none*	
			21 D	280	none	
8	21	3	22	370	none*	485
			23 K	370	+	
			24	370	none*	

D = died; K = killed.

\* Absence of tuberculosis determined by repeated tuberculin tests.

lung, which was the cause of death. Acid-fast bacilli were demonstrated in the cervical nodes. The route of infection here is largely enteric.

In Cage 6, all the three contacts developed tuberculosis. Guinea pig 16 was exposed for the entire period and died 467 days after the beginning of the experiment. The mesenteric nodes are fibrous and enormously enlarged, measuring 50 x 20 mm., and showed acid-fast bacilli in a smear. No tuberculous changes were seen anywhere else in the body; the cause of death was fibrinous pleurisy. The route of infection is unquestionably enteric.

Guinea pig 17 was exposed the entire period and died 440 days after the beginning of the experiment. The tuberculous mesenteric nodes are 7 in number and fibrocaseous, the largest measuring 24 x 12 mm.; the superficial and deep cervical nodes are extensively involved and fibrocaseous. The tracheobronchial and other lymph nodes are moderately affected. There is a generalized, rather chronic type of tuberculosis in the spleen, liver and lungs with excavation in the latter. Innumerable acid-fast bacilli were demonstrated in the lungs. The route of infection is largely enteric.

Guinea pig 18 was exposed for 266 days and died 379 days after the beginning of the exposure. There is a massive, fibrocaseous tuberculosis of the mesenteric nodes, which measure 35 x 12 mm. The mesenteric border of the intestines is extensively involved by a confluent nodular fibrocaseous tuberculosis. The cervical, and other lymph nodes are extensively tuberculous. The tracheobronchial nodes measure 20 x 14 mm. and are fibrocaseous. There is a massive, somewhat fibrous type of tuberculosis in the spleen, liver and lungs, with cavities in the latter. Numerous acid-fast bacilli were demonstrated in the lungs. The route of infection is largely enteric.

Guinea pig 19 of Cage 7 died 303 days after the beginning of the exposure. The mesenteric nodes are small and contracted, with fibrocaseous foci. The cervical nodes are similarly affected. The tracheobronchial nodes show a single minute caseous lesion on each side. There is an extensive tuberculosis of the spleen, fibrosis in the liver and, in the lung, numerous tubercles, in which acid-fast bacilli were demonstrated. The cause of death was pneumonia. The route of infection is doubtful.

In Cage 8, Guinea pig 23 developed tuberculosis. It was exposed for 370 days and was killed 485 days after the beginning of the experiment. The mesenteric nodes are normal. Two cervical nodes measure 10 x 6 mm. and 8 x 4 mm. Each shows caseous foci. The tracheobronchial lymph nodes are enlarged and contain questionable tubercles. There are a few discrete tubercles in the lung, liver and spleen. Acid-fast bacilli were demonstrated in a pulmonary nodule. The infection was probably acquired by way of the pharynx.

The remaining 17 guinea pigs, exposed for a period of 215 to 370 days, failed to develop tuberculosis. As in the experiments of the previous year no direct correlation could be established between the total number of inoculated animals in a cage and the incidence of contact tuberculosis. Thus in Cage 6 the three contacts

exposed to a total of 21 tuberculous cage mates all developed tuberculosis but in Cage 3 none of the contacts developed tuberculosis although they were exposed to 23 tuberculous cage mates for the same length of time.

Unfortunately, in the more crowded cages, both the ordinary and the special, tissue from some of the sources of contagion was eaten by their cage mates before their death could be discovered. The evidence obtained from these two groups must therefore be regarded as diminished in value, although there was no constant correlation between this accident and the development of tuberculosis, and the results were amply corroborated by those obtained with the other two groups, the guinea pigs confined 4 per cage in 12 ordinary and 12 special cages, in which no tuberculous tissue was eaten.

*Summary.*—Of 24 normal guinea pigs in groups of 3 exposed in ordinary cages to 3 tuberculous cage mates for an average period of 339 days, 7 or 29 per cent developed tuberculosis. Of these, 1 was killed 485 days after the beginning of exposure; 3 died of an intercurrent infection with tuberculosis, and 3 died of tuberculosis alone. The route of infection was largely or wholly enteric in 5, probably the pharynx in 1, and doubtful in 1.

The disease was chronic in nature. In 3 animals there was little if any spread beyond the portal of entry; in 3 it was slowly progressive with healing in the liver or pulmonary excavation and in 1 the disease, though generalized, lasted for 9 months, as indicated by the tuberculin test. The average survival of exposure of those that died with tuberculosis was 394 days, ranging between 303 and 467 days.

Where 3 normal guinea pigs were confined with 3 tuberculous animals in 8 special cages, in which the ingestion of faecal material was greatly reduced, 5 developed tuberculosis. The data are summarized in Table II.

Guinea pig 8 of Cage 3 died 278 days after the beginning of the experiment. The mesenteric nodes are of normal size but fibrous. The tracheobronchial nodes are matted together, measuring 25 x 13 mm. and 17 x 7 mm.; both masses are caseous and encapsulated. There is an extensive fibrous caseous pneumonia of both lungs with occasional cavities, conglomerate tubercles in the spleen, discrete fibrous tuberculosis with healing in the liver and a generalized affection of the lymphatic system. Acid-fast bacilli were demonstrated in the lung. The route of infection here is the respiratory tract.

Guinea pig 17 of Cage 6 died 369 days after the beginning of the exposure. There is an extensive generalized tuberculosis of the lung, liver, spleen and lymphatic system. The mesenteric and tracheobronchial nodes are both extensively

involved. The latter are considerably the larger, fibrous and cartilaginous in hardness; the mesenteric nodes are fibrocaceous. Acid-fast bacilli were found in

TABLE II

*The Incidence of Contact Tuberculosis in Special Cages Containing Three Normal and Three Tuberculous Guinea Pigs Each*

Cage No.	Total No. of inoculated animals in cage during experiment	Total No. of contacts during experiment	The three contacts that survived the longest period	Duration of exposure	Presence of tuberculosis in the contacts	Days survived by contacts that developed tuberculosis
1	14	6	1	370	none*	278
			2 D	240	none	
			3 K	309	none	
2	30	8	4 D	214	none	
			5 K	356	none	
			6 K	370	none	
3	14	10	7 D	118	none	
			8 D	278	+	
			9 K	288	none	
4	13	10	10 D	370	none	
			11 D	328	none	
			12 D	211	none	
5	10	10	13 D	176	none	
			14 D	342	none	
			15 K	370	none†	
6	27	4	16	370	none*	
			17 D	369	+	369
			18	289	+	289
7	19	7	19 D	163	+	163
			20 D	341	+	341
			21	370	none*	
8	17	11	22	370	none*	
			23 D	68	none	
			24	370	none*	

D = died; K = killed.

\* Absence of tuberculosis determined by repeated tuberculin tests.

† Absence of tuberculosis confirmed by animal inoculation and culture.

the lungs. The route of infection is probably both the respiratory and the digestive tracts.

Guinea pig 18 of the same cage died after 289 days of exposure. The mesenteric nodes are a matted, fibrocaseous mass 25 x 15 mm.; the ileo-coecal nodes are similarly affected. The tracheobronchial and other lymph nodes are moderately affected and fibrocaseous. There are isolated tubercles in the lung and spleen. The cause of death was acute dilatation of the stomach. Acid-fast bacilli were demonstrated in the periportal nodes. The route of infection here is largely enteric. It is noteworthy that tissues from several tuberculous animals were eaten by the occupants of this cage.

Two deaths from tuberculosis occurred in Cage 7. Guinea pig 19 died 163 days after the beginning of the exposure. The lungs are consolidated by a pervasive fibrocaseous nodular tuberculosis. There is an extensive tuberculosis of the spleen and a fibrous tuberculosis of the liver. The tracheobronchial nodes are slightly enlarged and fibrous. The mesenteric and cervical nodes are moderately enlarged and fibrocaseous. Acid-fast bacilli were demonstrated in the lungs. The route of infection here is largely enteric.

Guinea pig 20 died 341 days after the beginning of exposure with an extensive generalized tuberculosis of the lung, liver, spleen, and lymphatic system. Both the mesenteric and the tracheobronchial nodes are extensively involved and fibrocaseous. Acid-fast bacilli were demonstrated in the lung. Both routes of infection were involved. In this cage again, tuberculous animals were eaten. But the tuberculosis of Guinea pig 19 could not be ascribed to this fact, for the eating occurred first on the 145th day of exposure only 18 days before the death of this animal from an extensive tuberculosis.

The 19 remaining contacts, exposed from 68 to 370 days, failed to develop tuberculosis.

*Summary.*—Of 24 guinea pigs in groups of 3 exposed to 3 tuberculous cage mates in special cages with wire-mesh bottoms for an average period of 293 days, 5 or 20.8 per cent died with tuberculosis. The route of infection in these animals was divided: in some it was largely or wholly respiratory; in others it was largely alimentary.

The disease was progressive in nature with little or no tendency toward healing. In 4 the tuberculosis was of an extensive generalized character; in 1 of them there was slight pulmonary excavation, but the animal succumbed 89 days after the disease was acquired. In 1 only had the disease spread little beyond the portal of entry, which was clearly the alimentary tract. The average duration of life was 288 days, ranging between 163 and 369 days.

Thus in these two groups the incidence of acquired tuberculosis in



TABLE III

*The Incidence of Contact Tuberculosis in Ordinary Cages Containing Two Normal and Two Tuberculous Guinea Pigs Each*

Cage No.	Total No. of inoculated animals in cage during experiment	Total No. of contacts in cage during experiment	The two contacts that survived the longest period	Duration of exposure	Presence of tuberculosis in the contacts	Days survived by contacts that developed tuberculosis
1	8	2	1	370	none*	353
			2 D	353	+	
2	10	2	3	370	none*	493
			4 K	370	+	
3	7	2	5 K	370	none†	454
			6 D	370	+	
4	9	3	7 D	236	none	
			8	370	none*	
5	8	2	9	370	none*	
			10	370	none*	
6	7	2	11	370	none*	
			12	370	none*	
7	4	2	13	370	none*	
			14	370	none*	
8	8	2	15	370	none*	439
			16 D	370	+	
9	6	3	17 K	370	none	280
			18 D	280	+	
10	8	4	19	315	none*	
			20 D	348	none	
11	6	3	21	288	none*	
			22	370	none*	
12	7	2	23 D	355	none	
			24 K	370	none**	

D = died; K = killed.

\* Absence of tuberculosis determined by repeated tuberculin tests.

\*\* Absence of tuberculosis confirmed by animal inoculation.

† Absence of tuberculosis confirmed by animal inoculation and culture.

the special cages was less than in the ordinary cages. The route of infection in the ordinary cages was largely enteric; in the special cages it was sometimes respiratory, sometimes enteric and sometimes both. In the ordinary cages the disease was distinctly more chronic than in the special cages; the contacts that acquired tuberculosis in these survived on an average 106 days longer than those in the special cages.

As noted above, the value of the data obtained for both these groups is problematical because of the eating of tuberculous tissue in some of the cages. However parallel results, unconfused by accidents, were observed in the other two groups, in which 2 normal guinea pigs were exposed to 2 tuberculous guinea pigs in ordinary and in special cages. The above data are therefore reported here as corroborative rather than as direct evidence.

When 2 normal guinea pigs were confined with 2 tuberculous animals in ordinary cages, 5 developed tuberculosis. The data are summarized in Table III.

In Cage 1, Guinea pig 2 died at the end of 353 days of exposure. The mesenteric nodes measure  $27 \times 14$  mm. and are fibrocaseous. The tracheobronchial nodes are enlarged and fibrous. There is a generalized tuberculosis of a chronic type with pulmonary excavation. Numerous acid-fast bacilli were found in a smear from the lungs. The route of infection is probably both enteric and respiratory.

Guinea pig 4 of Cage 2 was exposed for 370 days and was killed 493 days after the beginning of the experiment. The mesenteric nodes are free of tuberculosis. The tracheobronchial and cervical nodes are enlarged and extremely fibrous. There are discrete tubercles in spleen, healed tuberculosis in the liver, and a small number of tubercles in the lung. Acid-fast bacilli were demonstrated in the tracheobronchial nodes. The route of infection is largely respiratory.

Guinea pig 6 of Cage 3 was exposed for 370 days and died 454 days after the beginning of the experiment. The cervical nodes are caseous; the mesenteric are enlarged and fibrous. The tracheobronchial nodes are negative. There is an extensive tuberculosis of the spleen, healing in the liver, and fibrous nodular pulmonary tuberculosis. The route of infection is alimentary.

Guinea pig 16 of Cage 8 was exposed for 370 days and died 439 days after the beginning of exposure. The mesenteric nodes are greatly enlarged and fibrocaseous, extending from the root of the mesentery to the hilum of the liver and measuring  $37 \times 12$  mm. The cervical nodes are enlarged and fibrocaseous. The tracheobronchial nodes are small and contracted. There is an extensive chronic generalized tuberculosis of the lung, liver and spleen, with exudation into the pleural and peritoneal cavities. The route of infection is enteric.

Guinea pig 18 of Cage 9 died 280 days after the beginning of exposure from hemorrhage into the peritoneal cavity. All the lymph nodes are free of any gross tuberculous changes. The spleen is enlarged, with pin-point tubercles. There is a massive tuberculous bronchopneumonia; a smear of the lungs shows a pure culture of acid-fast bacilli. The route of infection is probably respiratory.

The remaining 19 guinea pigs exposed for 236 to 370 days failed to develop tuberculosis.

*Summary.*—Of 24 normal guinea pigs in groups of 2 exposed to 2 tuberculous cage mates in ordinary cages for an average period of 353 days, 5 or 20.8 per cent developed tuberculosis. The route of infection was clearly enteric in some, in others respiratory.

The disease was of a chronic nature with fibrosis, marked healing in the liver and sometimes pulmonary excavation. The 4 animals that died with tuberculosis survived an average of 381 days, ranging between 280 and 454 days.

Where 24 normal guinea pigs in groups of 2 were exposed to 2 tuberculous cage mates in the special cages, 4 developed tuberculosis. The data are summarized in Table IV.

Guinea pig 1 of Cage 1 died on the 261st day of exposure. The mesenteric nodes are hardened. There is an extensive fibrocaseous tuberculosis of the tracheobronchial nodes. There is a generalized tuberculosis of the liver, spleen and lungs with two cavities of pin-head size in the latter. Acid-fast bacilli were demonstrated in the lungs. The cervical and retrosternal nodes are fibrocaseous. The route of infection here is largely respiratory.

Guinea pig 7 of Cage 4 died from tuberculosis 282 days after the beginning of the experiment with massive, fibrous tuberculosis of the tracheobronchial nodes, which measure 24 x 15 mm. The mesenteric and cervical nodes are moderately enlarged and fibrocaseous; the former measure 15 x 12 mm., the latter, 15 x 8 mm. There is a generalized tuberculosis of the spleen, liver and lungs. The route of infection is mainly the respiratory tract. Acid-fast bacilli were demonstrated in the lungs and cervical lymph nodes.

Guinea pig 10 of Cage 5 died on the 212th day of exposure. There is a massive, caseous encapsulated tuberculosis of the tracheobronchial lymph nodes, which measure 27 x 27 mm. The mesenteric nodes present a single fibrocaseous focus. There is an extensive generalized tuberculosis of fibrous type in the lung, liver, spleen and lymphatic system. The lungs contain acid-fast bacilli. The infection has invaded the animal by way of the lungs.

Guinea pig 14 of Cage 7 died after 313 days of exposure. The mesenteric and cervical nodes are normal. There is an extensive fibrocaseous tuberculosis of the tracheobronchial nodes, an extensive fibrous nodular tuberculosis of the lungs,

TABLE IV

*Incidence of Contact Tuberculosis in Special Cages Containing Two Normal and Two Tuberculous Guinea Pigs Each*

Cage No.	Total No. of inoculated animals in cage during experiment	Total No. of contacts in cage during experiment	The two contacts that survived the longest period	Duration of exposure	Presence of tuberculosis in the contacts	Days survived by contacts that developed tuberculosis
1	12	3	1 D 2 K	261 370	+ none	261
2	7	4	3 4	215 370	none* none*	
3	8	2	5 D 6 K	351 370	none none	
4	13	5	7 D 8 K	282 206	+ none**	282
5	6	3	9 10 D	370 212	none* +	212
6	9	4	11 K 12 D	266 335	none† none	
7	10	4	13 14 D	370 313	none* +	313
8	9	2	15 16 D	370 370	none* none	
9	12	6	17 18 D	370 255	none* none	
10	8	2	19 D 20	370 370	none none*	
11	6	2	21 K 22 K	370 370	none none†	
12	6	4	23 24 K	154 370	none* none**	

D = died; K = killed.

\* Absence of tuberculosis determined by repeated tuberculin tests.

\*\* Absence of tuberculosis confirmed by animal inoculation.

† Absence of tuberculosis confirmed by animal inoculation and culture.

healing in the liver, and chronic, discrete tuberculosis in the spleen. The route of infection is respiratory.

*Summary.*—Of 24 normal guinea pigs exposed in the special cages in groups of 2 to 2 tuberculous cage mates for an average period of 319 days, 4 or 16.6 per cent died of generalized progressive tuberculosis with an average duration of life of 267 days, ranging between 212 and 313 days. In all of them, the route of infection was largely the respiratory tract.

Again, as was seen above in the more crowded cages, the incidence of contact tuberculosis in the special cages was lower than in the ordinary cages, but the severity of the disease was greater, the average length of survival being 267 days, or 114 days less than in the ordinary cages, where guinea pigs acquiring tuberculosis by contact lived an average of 381 days. Again, as in the more crowded cages, enteric infection played a prominent rôle in the genesis of the disease acquired in the ordinary cages, whereas in the special cages the disease was predominantly of respiratory origin.

That this longer survival of exposure in the ordinary cages is not due to the fact that the infection was contracted in these cages later in the course of the experiment but actually to a more slowly progressive disease is indicated by the following facts. Half of the contacts in each group were subjected to repeated tuberculin tests, and it was found that when sensitiveness to tuberculin was once established it was never lost. On this basis, data as to the time of onset of the disease were available for 9 out of the total of 21 contacts that developed tuberculosis in all 4 groups. Of these 9 contacts, 5 were in ordinary cages and 4 were in special cages. One of the 5 in the ordinary cages died of pneumonia. Of the remaining 8, the 4 guinea pigs in the ordinary cages lived 270, 150, 290 and 251 days, or an average of 240 days after the appearance of a positive tuberculin reaction, and the 4 guinea pigs in the special cages lived 89, 71, 137, and 87 days, or an average of only 96 days after the appearance of the positive tuberculin reaction. It can be said with certainty therefore, that when the alimentary canal as a route of infection is practically eliminated from cage contagion, a more acutely fatal form of disease is then acquired, chiefly through the respiratory tract.

Of the 24 guinea pigs that served as controls for the tuberculous contagion of the room 3 died with tuberculosis. It will be remembered

that they were distributed 4 per cage, 16 in ordinary cages and 8 in special cages. Two of the 16 in the ordinary cages and 1 of the 8 in the special cages died with tuberculosis. Two of the 3 died, in a good state of preservation, of intercurrent disease with tuberculosis after 321 and 324 days of exposure. The third died of tuberculosis on the 317th day of exposure. All 3 presented essentially the same pathological appearances: extensive or massive fibrocaceous or caseous tuberculosis of the tracheobronchial nodes, little or no tuberculosis in the mesenteric nodes, and a generalized, chronic type of tuberculosis with marked healing in the liver. Acid-fast bacilli were demonstrated in each animal. The route of infection in all these was clearly respiratory.

TABLE V

*The Effect of Eliminating Enteric Contagion on the Incidence and Course of Tuberculosis Acquired by Normal Guinea Pigs Confined with Tuberculous Cage Mates*

Degree of crowding	Cage type	No. of guinea pigs exposed	Average duration of exposure	No. developing tuberculosis	Percentage developing tuberculosis	Average survival in days of contacts dying with tuberculosis
6 per cage, 3 inoculated, 3 normal	Ordinary	24	339	7	29	394
	Special	24	295	5	20	288
4 per cage, 2 inoculated, 2 normal	Ordinary	24	353	5	20	381
	Special	24	319	4	16	267

## SUMMARY AND DISCUSSION

In these studies normal guinea pigs were exposed to tuberculous cage mates in two different degrees of crowding, some in ordinary cages, where the food became contaminated with the excreta, laden with tubercle bacilli, of the inoculated animals, and some in special cages with wire-mesh floors, where these excreta were largely excluded as a source of contagion. The results are summarized in Table V.

In all these experiments crowding has always increased the incidence of the disease both in the ordinary and the special cages. However, due to the accident of eating of tuberculous animals in the more crowded cages these results are not conclusive, but they are in accord with the results of the previous study (2).

It was found that the incidence of tuberculosis was lower among the guinea pigs exposed to contagion in the special cages, in which little of the contaminated excreta remained in direct contact with the normal animals. In fact in the less crowded special cages the incidence of tuberculosis was only a little higher than among the same total number of guinea pigs exposed at the same time in the same room but not confined with tuberculous cage mates. In the special cages, especially in the less crowded, where nearly all the contaminated excreta were removed, the disease acquired was largely respiratory in origin and was characterized by an extensive tuberculosis of the tracheobronchial lymph nodes with little or no affection of the mesenteric nodes. The pulmonary disease in the guinea pigs that acquired tuberculosis in these cages was more often extensive than that acquired in the ordinary cages. However no definite evidence of primary lesions in the lung in the former was found, due to the dissemination of the disease at the time of death. The tuberculosis acquired by the contacts in the ordinary cages, on the other hand, was usually enteric in origin, although both routes were involved, especially in the less crowded cages.

Moreover, a striking fact appears if we compare the course of tuberculosis in these two types of cages in both degrees of crowding: the contacts that developed tuberculosis in the ordinary cages survived an average of 106 and 114 days longer than the corresponding animals in the special cages.

It is well known that the respiratory route is a far more dangerous avenue of infection in tuberculosis than the alimentary canal. This fact has again been clearly brought out in the recent studies of Bruno Lange and his associates (3), who have shown that although guinea pigs may occasionally be infected by way of the mouth with quantities as low as 0.000,001 mg. of virulent tubercle bacilli they often escape infection even when fed 0.1 mg. or more. On the other hand if guinea pigs are made to inhale even the smallest quantities of tubercle bacilli they regularly contract tuberculosis. Furthermore they have also shown that the alimentary infection produces a disease far more chronic in character than that acquired by the respiratory route. It would therefore seem that the more acute type of disease is acquired in the special cages because in these cages the respiratory route plays

by far the more important rôle whereas in the ordinary cages the disease is largely of enteric origin.

However, it appears that in the ordinary cages where the guinea pigs were constantly stirring up their sawdust or peat-moss bedding, laden with tubercle bacilli, there was an even greater opportunity for respiratory infection than in the special cages where there was no bedding at all and where most of the excreta were removed from immediate contact with the normal animals. That the respiratory mode of infection plays a rôle even in the ordinary cages was shown in a previous communication (2) as well as in this paper. In both studies it was found that only in the more crowded ordinary cages is the disease almost entirely of enteric origin but in the less crowded ordinary cages infection takes place by the respiratory tract as well, though to a smaller degree than by the alimentary.

It would therefore appear that in the ordinary cages both routes are open for infection but the relatively larger doses of tubercle bacilli ingested determine a disease of enteric origin and hence of a chronic nature, suppressing at the same time the engrafting of the disease by way of the lungs, and that in the special cages where the alimentary sources of infection have been reduced to a minimum, the disease is engrafted by way of the lungs and is therefore of more acute type. Although in the special cages the intensity of exposure even to tubercle bacilli entering by the respiratory tract is less than in the ordinary cages, nevertheless the disease so produced is more acute, presumably because the inhibitory or retarding effect of enteric infection upon the development of respiratory disease is absent.

This explains the paradoxical effect observed of a greater incidence and a more chronic type of disease in the ordinary cages and a lower incidence and a more acute type of disease in the special cages, for the intensity of exposure by both routes is greater in the ordinary cages, and of the two, much greater by the enteric route.

#### CONCLUSIONS

1. If normal guinea pigs are confined with tuberculous cage mates in cages where the food becomes contaminated with the excreta, laden with tubercle bacilli, of the inoculated animals, the incidence of acquired tuberculosis amongst them is greater than amongst guinea



pigs similarly exposed in cages where this mode of infection is largely eliminated.

2. The disease acquired in the first type of cage is largely of enteric origin and is chronic in type.

3. The disease acquired in the second type of cage is of respiratory origin and has a more acute course.

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# EXPERIMENTAL EPIDEMIOLOGY OF TUBERCULOSIS

## THE ROUTE OF INFECTION IN NATURALLY ACQUIRED TUBERCULOSIS OF THE GUINEA PIG

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It is generally held that the route of infection of tuberculosis in man is, with infrequent exceptions, the lungs and the alimentary tract, the latter including the pharynx and its lymphatic structures. Numerous experiments more or less reproducing the supposed modes of infection by these routes in man have been performed, either by feeding animals tubercle bacilli or by introducing droplets, air or dust laden with tubercle bacilli more or less directly into their lungs. The disease produced thus has been characterized by extensive lesions of the cervical and mesenteric nodes, when the mode of infection has been alimentary and by lesions in the lungs and the tracheobronchial lymph nodes when the respiratory route has been used. For a review of the literature and renewed experimental studies in this field the reader is referred to the recent publications of B. Lange and his associates (1).

In the studies of the experimental epidemiology of tuberculosis conducted in the laboratory of The Henry Phipps Institute tuberculosis was acquired by normal guinea pigs living in the same cage or in the same room with tuberculous animals under conditions more nearly approaching natural infection in man. In these experiments 45 guinea pigs have acquired tuberculosis under different conditions of exposure. The detailed observations on each of these animals have been recorded in other papers (2). It seemed desirable to compare the type of lesion produced in these animals by the different modes of exposure.

Of these 45 guinea pigs, 27 have acquired tuberculosis by being exposed to tuberculous cage mates under the following conditions. Eleven were exposed in groups of 3 to 3 tuberculous cage mates in

"ordinary" metal cages with a pan as floor, upon which the food was placed and came into contact with the bacilli-bearing excreta from the tuberculous animals. Seven have acquired tuberculosis in the same type of cage under the same conditions except that they were less crowded, groups of 2 being exposed to 2 tuberculous cage mates. Five guinea pigs have acquired tuberculosis by being exposed in groups of 3 to 3 tuberculous cage mates in "special" cages, which differed from the ordinary cages in that the floor was  $\frac{1}{4}$  inch wire-mesh, through which the faecal boluses fell into a pan lying 3 inches below. The food for these animals was placed in metal cups attached to the door and walls of the cage. Four guinea pigs were exposed, like the last group, in special cages, but were less crowded, groups of 2 being confined with 2 tuberculous cage mates. Eighteen guinea pigs have acquired tuberculosis by being exposed to tuberculous animals in the same room but not in the same cage.

These experiments have extended over the past 3 years. Of the 45 animals, the mesenteric and tracheobronchial lymph nodes of 35 were available for comparison.

In Fig. 1 the relative size of the tuberculous mesenteric and tracheobronchial nodes of each of these animals has been depicted by tracing their borders. They are grouped according to the conditions of exposure under which the tuberculosis was acquired.

It will be seen that the tuberculosis acquired by guinea pigs exposed to tuberculous cage mates in the more crowded ordinary cages, where the food was soiled with tubercle bacilli, is characterized by a great enlargement and an extensive tuberculosis of the mesenteric nodes. The tracheobronchial lymph nodes, on the other hand, are involved to a very much smaller degree. Occasionally, as in Guinea pig 1, the disease is limited to the mesenteric nodes. In no case is the disease of the tracheobronchial nodes equal to that of the mesenteric and, on an average, the latter are on cross section 3.6 times larger than the former. Here the route of infection is predominantly enteric although the affection of the tracheobronchial nodes would suggest that the respiratory route is not completely eliminated. It would appear that under these conditions of exposure tubercle bacilli penetrate into the system by both routes but the more intensive exposure to infection by way of the intestinal tract determines the more extensive tuberculosis of the mesenteric nodes.

Where guinea pigs were exposed under identical conditions but in less crowded ordinary cages the size of the mesenteric lymph nodes is again very much larger than that of the tracheobronchial nodes, but

6 Animals in Ordinary Cages 3 infected - 3 normal			4 Animals in Ordinary Cages 2 Infected - 2 normal			6 Animals in Special Cages 3 Infected - 3 normal			4 Animals in Special Cages 2 Infected - 2 normal			Exposed to tuberculous Animals in Pen but not in Same Cage		
Animal	Mesenteric	Tracheo- Bronch	Animal	Mesenteric	Tracheo- Bronch	Animal	Mesenteric	Tracheo- Bronch	Animal	Mesenteric	Tracheo- Bronch	Animal	Mesenteric	Tracheo- Bronch
1		no tbc	10			15			20			24	no tbc	
2			11			16			21	no tbc		25		
3			12			17			22			26	no tbc	
4			13			18			23			27		
5			14			19						28	no tbc	
6												29	no tbc	
7												30	no tbc	
8												31		
9												32	no tbc	
												33		
												34		
												35	no tbc	

FIG. 1. The relative size of the mesenteric and tracheobronchial lymph nodes in contact tuberculosis of guinea pigs acquired under different conditions of exposure

the difference between the two groups of nodes is less marked, the mesenteric being 2.3 times instead of 3.6 times larger, as in the guinea pigs that acquired tuberculosis under more crowded conditions. In 2

out of the 5 guinea pigs, the extent of the disease is approximately the same in both groups of nodes. One may therefore say that in the less crowded ordinary cages the route of infection is again predominantly enteric but partly, and to a somewhat greater extent, respiratory.

Where guinea pigs were exposed in special cages with wire-mesh floors, to tuberculous animals, the excreta of which were largely eliminated from contact with the normal animals, the relative involvement of these two groups of nodes was reversed, namely, the tracheobronchial nodes were in some much more extensively tuberculous than the mesenteric. In 2 of the 5 guinea pigs that acquired tuberculosis in the very crowded special cages, the mesenteric nodes were involved to a greater extent than the tracheobronchial nodes; in one the affection was equal and in 2 the tracheobronchial nodes were affected to a greater degree. It is noteworthy that in these crowded cages considerable amounts of faecal material tended to become aggregated in the corners of the cage.

Thus in comparison with the tuberculosis acquired by cage mates exposed in similarly crowded ordinary cages, the disease acquired by the guinea pigs exposed in special cages was to a much greater extent of respiratory origin, although in some of the contacts in this group the disease was frankly enteric. Unfortunately, the results obtained with some of the more crowded cages, both the ordinary and the special, were complicated by the eating of tissue from dead tuberculous animals by the cage occupants. Nevertheless even in comparison with the tuberculosis acquired in the less crowded ordinary cages, where no eating of tuberculous tissue occurred, the disease acquired in the crowded special cages was still to a greater extent of respiratory origin.

Thus with the partial elimination of the enteric route of infection the respiratory route comes into prominence. This fact is more clearly brought out in the less crowded special cages where no eating of tuberculous animals occurred and where the contaminated excreta were almost completely removed. Each of the 4 guinea pigs that acquired tuberculosis in these showed a far greater affection of the tracheobronchial nodes than of the mesenteric. In one there was no tuberculosis of the mesenteric nodes in the presence of an extensive tuberculosis of the tracheobronchial; in another the affection of the former nodes

was negligible in comparison with the massive disease of the latter, and in 2 although the disease of the mesenteric nodes was considerable that of the tracheobronchial nodes was more extensive. On the average, the tracheobronchial nodes were 4 times larger than the mesenteric, a relationship directly the opposite of that seen in the crowded ordinary cages. Thus with a greater reduction in the intensity of exposure to enteric infection the bacillus penetrates largely by way of the respiratory tract.

The pulmonary disease in the guinea pigs that acquired tuberculosis in the special cages was more often extensive than that acquired in the ordinary cages. However no definite evidence of primary lesions in the lung in the former was found due to the dissemination of the disease at the time of death.

Where normal guinea pigs are kept in the same room but not in the same cage with tuberculous animals the tracheobronchial nodes in every case are massively affected. In 7 out of 12 animals that acquired tuberculosis under these conditions the mesenteric nodes were entirely free of macroscopic tuberculosis. In 4 there was a slight affection of the mesenteric nodes but a massive tuberculosis of the tracheobronchial lymph nodes. Here one may say that the route of infection is almost entirely by way of the respiratory tract and the amount of possible enteric infection, such as may be carried by the animal attendant from cage to cage, is negligible.

Associated with these lesions in the tracheobronchial lymph nodes, there was usually an extensive tuberculosis in the lungs frequently with excavation. In some there were lesions closely resembling human tuberculosis of the childhood type, one or several small nodules in the lung being accompanied by massive caseated tracheobronchial lymph nodes. This observation was possible as some of the animals died from intercurrent disease before the tuberculosis had become disseminated.

It is now generally accepted that tubercle bacilli may penetrate the intestinal mucosa without setting up any lesion at the site of entry, but usually the draining lymph nodes become extensively tuberculous. Amongst the guinea pigs that acquired tuberculosis by the enteric route in only 2 out of 27 was there any specific ulceration of the intestines, as revealed by a gross examination of the mucosa of the ileum

and that of the large intestines, and occasionally even the local lymph nodes failed to become tuberculous, as in Guinea pig 5. When tuberculosis develops by way of the respiratory route the lung is nearly always affected, but even here the tracheobronchial lymph nodes may be the seat of extensive tuberculosis without any macroscopic tuberculosis of the lung. Occasionally, as in Guinea pig 34, the reverse may be true.

#### SUMMARY AND DISCUSSION

Under conditions closely simulating the natural modes of tuberculous infection in man normal guinea pigs have acquired tuberculosis by being exposed under two degrees of crowding to tuberculous cage mates in ordinary cages, where the food became soiled with excreta, bearing tubercle bacilli, and in special cages, with wire-mesh floors, where this source of infection was almost entirely eliminated. Guinea pigs were also exposed in the same room but not in the same cage with tuberculous animals. It was found that the relative tuberculous involvement of the mesenteric and tracheobronchial nodes showed a gradation of change from an almost completely alimentary infection to a completely respiratory infection. The disease involved the mesenteric nodes predominantly in the crowded ordinary cages, with much less or no affection of the tracheobronchial nodes. It was similarly, but less markedly, enteric in origin in the less crowded ordinary cages, the mesenteric nodes again being larger than the tracheobronchial nodes, but the difference in size was not so great. In the more crowded special cages the relative affection of these two groups of nodes alternated, so that in some the mesenteric, in some the tracheobronchial nodes were more extensively tuberculous. A disease characterized by less or no affection of the mesenteric nodes and by extensive lesions of the tracheobronchial nodes was seen in the less crowded special cages. Finally there was a massive tuberculosis of the tracheobronchial nodes with usually no affection of the mesenteric nodes in the frankly air-borne tuberculosis acquired by guinea pigs exposed in the same room but not to tuberculous cage mates.

This gradation in the rôle played by the enteric and respiratory routes of infection, as first the one and then the other becomes the more frequent channel of entrance for tuberculosis, would indicate

that the penetration of tubercle bacilli by the one portal of entry inhibits the engrafting of tuberculosis in the tissues by way of the other portal of entry. It is apparent that in the special cages the opportunities for inhaling tubercle bacilli are at most equal to if not much less than in the ordinary cages; for in the latter dust from the bedding, laden with tubercle bacilli, is stirred up almost constantly by the animals, whereas in the special cages there is no bedding at all, and therefore, presumably, no more tubercle bacilli in the air than may occur in any part of the room. Nevertheless the route of infection was predominantly the respiratory tract in the special cages, especially in the less crowded, apparently because the enteric route had been largely eliminated. The greater predominance of the respiratory route amongst guinea pigs that acquired tuberculosis in the less crowded ordinary cages as compared to the lesser significance of this route in the more crowded ordinary cages would point in the same direction. These observations are in harmony with our knowledge that tuberculosis once implanted in an organism confers a certain degree of immunity to the disease. It is noteworthy that in a study of human autopsy material Opie (3) has found that when healed lesions are present in the mesentery focal tuberculosis in the lungs is seldom found, and that when first infection occurs by way of the lungs it tends to prevent the engrafting of the disease by way of the intestinal tract.

#### CONCLUSIONS

1. In tuberculosis of guinea pigs acquired by contact with tuberculous guinea pigs under conditions permitting the entrance of tubercle bacilli both by way of the alimentary and of the respiratory tracts, the type of lesion produced depends upon the relative intensity of exposure to infection by one or the other channel.

2. With the gradual elimination of exposure to alimentary infection tuberculosis is more and more completely engrafted through the respiratory route.

3. With the gradual increase in the intensity of exposure to alimentary infection, the disease becomes more and more completely enteric in origin.

4. Some evidence is presented that the engrafting of tuberculosis by



way of the alimentary route inhibits the development of respiratory disease.

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# EXPERIMENTS WITH THE VIRUS OF POLIOMYELITIS\*

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## *I. Attempted Production of Poliomyelitis in Rabbits*

The majority of the experiments reported in this communication are concerned with the attempted production of poliomyelitis in rabbits.

Since Landsteiner and Popper produced acute anterior poliomyelitis in monkeys in 1909, attempts to produce the disease in rabbits have been numerous. The results in most instance have been entirely negative although several investigators have reported paralyzes and typical or atypical lesions through several generations of young rabbits. Some recent workers, Fairbrother (1) and Harmon, Shaughnessy and Gordon (2), give comprehensive summaries of the literature, making an extended review unnecessary here. Fairbrother reported his own negative results with intracerebral, intravenous and intraperitoneal injections of young rabbits and his inability to adapt the virus to the rabbit by brain passage. Harmon, Shaughnessy, and Gordon using intraperitoneal and intracerebral injection of young rabbits also had completely negative results.

The ease with which the viruses of vaccinia (Noguchi (3)) and of herpes (Levaditi (4), Gay and Holden (5)) are adapted to the testicle of the rabbit suggested to us that testicular injection and passage might be a favorable method for adapting the poliomyelitis virus to the rabbit organism. The apparent symbiosis of vaccinia and poliomyelitis viruses in the monkey's skin reported by Thomsen (6), suggested an additional factor which might possibly aid in the adaptation.

The following experiments were carried out combining these two suggested aids to adaptation.

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Rabbits of about 1500 to 2000 gm. were used for the injections and passages, except in one series of experiments where very young rabbits were used as indicated below. Small or medium sized *Macacus rhesus* monkeys were used. The poliomyelitis virus used is a moderately virulent strain supplied by Dr. Aycock. The virus, as injected, consisted of the supernatant of a centrifugalized 20 per cent saline sand-ground suspension of monkey cord (less often brain) obtained in the first few days of paralysis. The vaccine virus was a 20 per cent centrifugalized suspension of a Noguchi testicular virus harvested at 4 days.

Attempts were first made to produce poliomyelitis in the rabbit by direct testicular injection of poliomyelitis virus suspension and of mixtures of equal parts of poliomyelitis and vaccine viruses. 1 cc. of the poliomyelitis virus suspension was injected into both testicles of ten rabbits; from eight of which the testicles were removed at periods varying from 1 to 7 days for further passages. In two the testicles were left undisturbed. Locally only a moderate inflammatory reaction occurred. Symptoms occurred in only one animal—a paralysis of the forelegs on the seventh day. The cord of this animal showed only patches of round cell infiltration which were present also in control cords. Three rabbits were injected into each testicle with 2 cc. of the mixture of equal parts of poliomyelitis and vaccinia viruses. None of these animals developed any symptoms; the testicles showed only the usual vaccinal reactions.

Attempts were made to adapt the virus to the rabbit by serial passage through several testicles with final injection into the brain.

20 per cent virus emulsion was injected into a rabbit's testicle which was removed aseptically under anaesthesia in 4 days, ground up with saline and reinjected into the testicle of a new animal. The virus was passed in this manner through four animals. The testicle of the fourth animal was injected into the 4th ventricle of a fifth. No symptoms occurred in the final animal or in any animal of the series. A similar series with final intracerebral injection was also negative. A serial testicular passage through four animals at 3 day intervals in which each injected testicle was injected intracerebrally into a test animal also gave entirely negative results. Mixtures of equal parts of poliomyelitis and vaccinia viruses were tested for adaptation in this same manner in two series without development of symptoms in any animal. The vaccinia virus used had been previously shown not to produce encephalitis.

Incidental to the attempted production of immune serum reported below, a number of rabbits were given repeated combined intraperitoneal and intradermal injections of poliomyelitis virus and of poliomyelitis and vaccine virus mixtures.

Six were given ten 1 cc. intraperitoneal injections and ten 0.1 cc. intradermal injections of poliomyelitis virus during a period of 5 weeks. One rabbit was given twenty such combined injections during 10 weeks. No paralyzes or significant symptoms occurred in any.

In a second group nine rabbits were given ten 2 cc. intraperitoneal and ten 0.2 cc. intradermal injections of the poliomyelitis-vaccine virus mixtures. One was given twenty such injections. No significant symptoms occurred in this group.

Since most of the reported successful attempts to produce the disease in rabbits have been made with animals somewhat younger than those used in the above work, a number of experiments were carried out using animals weighing from 500 to 800 gm.

Three animals were injected intracerebrally with 0.3 cc. of a mixture of equal parts of 20 per cent poliomyelitic monkey cord and 20 per cent normal rabbit testicle. The normal testicle was added to the virus because of the apparent enhancement of the virus in monkeys by this tissue as reported below, and because of the enhancement of vaccine virus by testicular tissue reported by Duran-Reynals (11). One of this group showed salivation and a spastic condition of the hind leg in 28 days but recovered. The other two died within 30 days without definite symptoms.

At different times 20 per cent poliomyelitic monkey cord was injected into the testicles of three adult rabbits. These were removed in 24 hours, ground and injected intracerebrally into three groups of young animals in 0.2 and 0.3 cc. amounts. A total of 14 animals were injected in the three groups. Of these one showed definite flaccid paralysis of the hind legs in 28 days. An emulsion of the brain and cord of this animal injected intracerebrally into a monkey produced no symptoms. Three of the animals, two of which died, showed other symptoms including spastic leg conditions, salivation, convulsions and postural abnormalities. The brain and cord of one of the animals showing salivation and convulsions produced no symptoms when injected intracerebrally into a monkey. Seven of the animals died without symptoms, other than diarrhea, at periods from 5 to 27 days. The brain and cord of one of these injected into a monkey produced no symptoms. The remainder of the animals were still living and showed no symptoms after 2 months.

The brains and cords of some of the above animals showing definite symptoms were injected intracerebrally into seven young rabbits. Of these two showed spastic leg conditions at 13 and 28 days respectively. Two died without showing symptoms at 21 and 30 days. Three survived 2 months without symptoms.

Brains and cords of animals having shown symptoms were also passed through the testicles of an adult rabbit for a 24 hour period and again injected intracerebrally into young animals. Four were injected, none of which showed symptoms. Two died without symptoms at 3 days and 23 days.

A control group of young rabbits consisting of seven injected intracerebrally with normal rabbit testicle and five uninoculated was observed through the same period as the above. Many of the symptoms seen in the injected group were seen in the control group. Deaths without observable symptoms were just as numerous in this group—in fact only one animal survived of the seven inoculated with normal testicle. The only differences between the two groups were: first, definite flaccid paralysis occurred in the experimental group which never occurred in the control group; and second, the possibly greater proportion of definite symptoms to the number of deaths without symptoms seen in the virus injected group.

These results in young rabbits, especially in regard to the occurrence of symptoms in control animals, are in striking agreement with the results just reported by Harmon, Shaughnessy and Gordon.

To exhaust the possibilities of producing the disease in rabbits, the gastro-intestinal route of infection was tried.

Three rabbits were given 8 cc. doses of 20 per cent poliomyelitis virus by stomach tube on 2 successive days. In one of these a sterile irritation of the meninges was produced by the injection of 0.3 cc. broth into the 4th ventricle at the time of the second virus dose. Since Burnet and Conseil (7) found that chloral or opium injections increase the susceptibility of the brain to vaccine virus, another was given large subcutaneous injections of injectable opium with each virus dose. No symptoms were produced in any of these animals.

Although no disease could be produced in rabbits by testicular injection and passage the survival time of the virus in the rabbit's testicle was determined.

In other rabbit tissues the virus has been found to survive varying lengths of time: subcutaneous tissues several days, (Flexner and Clark (8)); anterior chamber 23 days, (Levaditi and Danulesco (9)) and in the brain 4 days but not 7, (Amoss (10)). 1 cc. of a 20 per cent poliomyelitic cord emulsion was injected into a rabbit's testicle and 1 cc. of an emulsion of this testicle made with 5 cc. saline 24 hours later, was injected intracerebrally into a monkey and produced no symptoms. This procedure was repeated with negative results on three occasions. That the virus was not destroyed by the tissue *in vitro* was shown by injection of a mixture of virus with fresh rabbit testicle in the same proportions. There was an apparent enhancement of the virus action by this mixture similar to that noticed by Duran-Reynals (11) with vaccinia. In spite of this enhancing action, the virus does not survive 24 hours in the testicle. Testicles injected 4 days previously with poliomyelitis virus also gave negative results on injection into monkeys. Similar experiments with poliomyelitis-vaccinia virus mixtures gave no evidence that the vaccine virus lengthened the period of survival.

While poliomyelitis virus causes no disease in rabbits and does not retain its pathogenicity for monkeys when passed rapidly through rabbits' testicles, it was considered possible that the virus so treated might retain its antigenic properties. Virus passed through 2, 3, and 4 rabbits' testicles at 4 day intervals, and repeatedly injected intraperitoneally and intradermally into monkeys produced no immunity. Rabbits' testicles injected with virus from 1 to 7 days previously, injected into 3 monkeys intraperitoneally and subcutaneously in 6 injections at 3 day intervals, gave no immunity. Similar experiments with poliomyelitis-vaccinia mixtures were likewise negative.

## *II. Attempted Production of Poliomyelitis Neutralizing Antibodies in Rabbits*

Attempts were next made to produce poliomyelitis neutralizing antibodies in rabbits by the injection of poliomyelitis virus suspensions and of poliomyelitis-vaccinia mixtures. While horses and sheep have been used rather frequently in attempts to produce specific serum, the only previously recorded attempt with rabbits is that of Tsen (12) who was unable to obtain any evidence of a neutralizing antibody in the serum of rabbits given repeated subcutaneous injections of poliomyelitis virus.

In our experiments three series of rabbits were given repeated intraperitoneal and intradermal injections of poliomyelitis virus and of poliomyelitis-vaccinia mixtures. In the first series three rabbits were given 10 combined intraperitoneal and intradermal injections of poliomyelitis cord during a period of 5 weeks—1 cc. intraperitoneally and 0.1 cc. intradermally; 3 were given 10 injections of poliomyelitis-vaccinia mixtures—2 cc. intraperitoneally and 0.2 cc. intradermally, and 3 controls were immunized only with vaccinia virus. The rabbits were bled from the heart 14 days after the last injection and the sera from each group pooled and tested for neutralizing antibodies. 0.5 cc. serum was mixed with 0.5 cc. 5 per cent virus supernatant, incubated 2 hours at 37°C. and left in the ice box over night. The mixtures were then injected intracerebrally into monkeys. Contradictory results were obtained with two separate tests of the sera of the first series. In one case the serum from the rabbits immunized by the poliomyelitis-vaccinia mixtures protected and the serum from the rabbits immunized by the poliomyelitis alone did not; in the other test the results were reversed. On account of these irregular results, two more series of rabbits were immunized. Five were given 10 intraperitoneal and intradermal injections of poliomyelitis suspensions in the same doses as before

and 7 were given the 10 injections of the virus mixtures as before. Two other rabbits were given 20 injections over a period of 10 weeks—one of poliomyelitis suspensions and the other of poliomyelitis-vaccinia mixture. Neutralization tests on the sera from these two series were carried out at the same time. The sera from the larger series were pooled into small groups and a total of 9 monkeys tested by the injection of serum plus 5 per cent virus mixtures from the two series.

None of the sera in these later series showed any neutralizing power—all the monkeys succumbing to typical poliomyelitis.

Despite the irregular results with the first two tests, it seems certain that an anti-poliomyelitis serum cannot be produced in the rabbit. Definite precipitin reactions have been obtained with normal and poliomyelitis monkey brain material with these sera and it is possible that the irregular apparent neutralizing action is due to the fixation of virus by this precipitation.

### *III. Infection and Immunization of Monkeys by the Gastro-Intestinal Route*

Recent reports of probable milk-borne epidemics by Aycock (13) and the epidemiological studies by Kling (14) indicating that the disease may be water-borne, have made it desirable to reconsider the question of infection by the gastro-intestinal tract. Schultz (15) has recently reported negative results by feeding infected milk to monkeys and has given a brief summary of the literature. Landsteiner and Levaditi (16) were unable to immunize by the gastro-intestinal route.

In our first experiment a small monkey after fasting 18 hours was given 15 cc. of bile by stomach tube. 24 hours later 15 cc. of 20 per cent poliomyelitis virus were given by the same method and at the same time an irritation of the meninges was produced by the injection of 0.3 cc. sterile saline into the 4th ventricle. No symptoms developed. 51 days later the bile and virus injections were repeated to determine if any sensitization had developed—likewise without results. 23 days after this injection the animal was tested for immunity by intracerebral injection and developed a typical, though extremely mild, poliomyelitis. The control developed a severe, fatal type of the disease.

In a second experiment three monkeys were given 4 intrastomachic injections of 10 cc. of 20 per cent virus emulsion preceded 24 hours previously by 10 cc. of ox bile. All the injections were made after at least 18 hours fasting and no food was given for an hour afterwards. The 4 injections were made during a period of 18 days. One monkey died 12 days after the last inoculation as a result of parasitic infestation, and showed no poliomyelitis symptoms or lesions in the cord.

The two remaining monkeys showed no symptoms and were tested for immunity by intracerebral injection of 1 cc. of 20 per cent virus cord 20 days after the last stomach injection. Neither showed any symptoms although three other monkeys inoculated at the same time with the same virus succumbed to severe typical poliomyelitis. On a second test inoculation of 1 cc. of 20 per cent virus 26 days after the first, one of the monkeys developed typical poliomyelitis in 8 days, the other showed only a mild and very much delayed weakness of the legs and still survives without paralysis 42 days after the test inoculation. In this case again several other animals inoculated at the same time with equal amounts of the same virus succumbed to rapid typical poliomyelitis.

The attempts to infect by the gastro-intestinal route were decisively negative in spite of irritation of the tract by bile. Whether or not immunization can be produced by this route is not so decisive, but these experiments give at least a strong suggestion that this may be possible.

#### *IV. No Concentration of Virus in the Cellular Elements of the Blood*

The virus of poliomyelitis has not been regularly found in the blood stream of monkeys after the usual methods of infection. Clark, Fraser and Amoss (17) were able to infect by the intracerebral injection of 4 cc. of defibrinated blood taken at the beginning of paralysis on the seventh day after intracerebral injection, but this was in only one case out of ten, and other workers have reported negative or only occasionally positive results. Recently Smith (18) has reported that vaccinia virus may be detected more constantly in the blood stream by separating the white cells, to which the virus is fixed, from the antibody-containing serum. An attempt was made to determine if this is also true for poliomyelitis.

Blood was taken from the hearts of several monkeys at various stages of infection with poliomyelitis. Heparin was added and the buffy coat separated by slow centrifugalization. The white cells from 10 cc. samples of blood, in all cases mixed with a very large proportion of red cells, were injected intracerebrally into monkeys. Blood was taken from three different monkeys at four different stages—from the first appearance of tremors on the seventh day after injection to the stage of final prostration and complete paralysis 11 days after injection. Intracerebral injection of the cells from these four samples into four monkeys produced no symptoms in any, giving no evidence that virus is present in any greater amount in concentrated cell suspensions than in whole blood.



*V. Immunization by Neutralized Virus*

Römer and Joseph (19) reported that an intracerebral injection of neutralized virus protected against subsequent injections, but Flexner and Lewis (20) were not able to produce immunity by neutralized virus.

A monkey was given 1 intracerebral injection of 1 cc. of a mixture of equal parts of convalescent monkey serum and 5 per cent virus and 4 subcutaneous injections of a like amount of convalescent serum and 20 per cent virus during a period of 30 days. An intracerebral injection of 1 cc. of 20 per cent virus 10 weeks after the last injection produced typical acute poliomyelitis, giving no evidence of any immunization by the serum-virus mixtures.

## SUMMARY

1. Efforts to adapt the virus of poliomyelitis to the rabbit organism and to produce poliomyelitis in rabbits by testicular injection and by brain injection after testicular passage produced no evidence that the virus could be adapted in this manner. Suggestive symptoms produced in very young rabbits were duplicated in non-specifically treated and in uninoculated controls. The admixture of a vaccine virus, adapted to the rabbit organism, with the poliomyelitis virus in similar injections and passages did not aid the adaptation. The virus of poliomyelitis did not survive 24 hours in the rabbit testicle—whether alone or mixed with vaccine virus.

Repeated intraperitoneal and intradermal injection of poliomyelitis virus and of poliomyelitis and vaccinia virus mixtures produced no disease in rabbits. Massive doses of concentrated virus by stomach tube in conjunction with meningeal irritation produced no symptoms in rabbits.

2. No neutralizing substances against poliomyelitis virus could be produced in rabbits by the repeated intraperitoneal and intradermal injection of poliomyelitis virus or of poliomyelitis-vaccinia virus mixtures.

3. Although attempts to infect monkeys by intrastomachic injections, after bile irritation of the mucosa, were entirely negative, evidence was obtained that repeated intrastomachic injection after bile irritation may produce an appreciable degree of immunity.

4. No evidence could be obtained that the cellular elements of the blood contain the virus in any greater proportion than the whole blood.

5. One attempt to immunize by neutral virus-serum mixtures was entirely negative.

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# METHODS FOR THE PURE CULTURE OF CERTAIN PROTOZOA

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PLATE 13

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Protozoology has been seriously hindered within recent years by a paucity of technical methods for obtaining pure cultures. The present work had its inception in attempts to grow and purify certain parasitic protozoa found in the alimentary tract of insects. However, because conditions appeared simpler with the free-living protozoa, these have been largely used.

It is known that certain trypanosomes, species of *Leishmania* and their related insect flagellates can be propagated in pure culture, and that bacteria when present may kill the protozoa by their toxic products. Bacteria outgrow and destroy many intestinal protozoa, that have so far resisted purification, and cultures can be maintained only by transferring every 2 or 3 days. It is generally agreed that bacteria are essential for some of the free-living, coprozoic and intestinal protozoa as food supply. Cutler and Crump (1) stated that *Colpidium* cultures containing few bacteria showed low rates of division and manifested definite signs of nutritional deficiency, whereas cultures with numerous bacteria appeared normal and had a very high division rate.

Because workers have found it impossible to free most protozoa from bacteria or to propagate them after they were free, some of their attempts have been confined to inhibiting the bacterial flora. *Euglena gracilis* tolerates an amount of citric acid which inhibits bacterial growth. This fact was used to advantage by Zumstein (2). Barret and Yarbrough (3) were able to inhibit bacteria in cultures of *Balantidium coli* by transferring the protozoa to sterile media every second day. Amster (4) showed that bacterial growth was suppressed in cultures of *Balantio-phorus species?* by using a dilute peptone solution at pH 6.8 and maintaining cultures at 22° to 25°C. Since egg albumen is known to possess bactericidal properties, Tanabe (5) incorporated it in media used for protozoa cultures. Kofoed and Johnston (6) have recently used dyes in combination with an egg albumen medium to rid cultures of *Endameba gingivalis* of many harmful bacteria.

Other investigators strove towards pure cultures and in a few cases were successful. Plating methods proved disappointing, because most protozoa do not colonize on solid media, and when colonies are formed bacteria are frequently included in them. However, some species of *Englena* do colonize on solid media, and these have been isolated from such colonies and grown in the pure state (Pringsheim, Mainx (7)). Pure cultivations from single cell isolations of *Englena* have also been reported by Ternetz (8); and Barber's cell isolation technique was successfully used by Noguchi (9) to obtain pure cultures of certain *Herpetomonads* parasitic in insects.

Animal inoculations to free protozoa from bacteria have been rarely used. *Herpetomonas muscae-domesticae* was freed by inoculating dilutions of the alimentary tract contents of parasitized house flies into the peritoneum of silkworms (*Bombyx mori*). Blood from some of the surviving silkworms was inoculated into media and yielded pure cultures of the protozoan (Glaser (10)).

Several methods for washing protozoa free from bacteria have been described (Ogata, Nöller, Parpart and Cleveland (11)). Parpart succeeded in obtaining bacteria-free *Paramecia*, and Cleveland obtained large numbers of sterile *Tritrichomonas fecalis*, but the cleansed protozoa did not grow in sterile media. Nöller, on the other hand, was not only successful in freeing *Trypanosoma melophagium*, parasitic in the gut of *Melophagus ovinus*, from bacteria, but he obtained pure cultures of the protozoan.

Ogata's technique, devised in 1893, was unknown to us until the completion of our work. It corresponds to one of the methods devised by us, and because of neglect, deserves reemphasis. Ogata filled sterile capillary tubes, of 0.3 to 0.5 mm. bore and 10 to 20 cm. long, by capillarity, with a sterile medium to within 1 to 2 cm. of the end. He then inserted them into a contaminated culture of the free-living ciliate *Polytoma uvella* and allowed them to fill completely, without air bubbles between the layers. Both ends were next sealed by heat. Within 5 to 30 minutes some of the ciliates were found within the sterile portion of the medium, having carried no bacteria with them. The upper parts of the tubes were broken and media inoculated. The cultures were held at room temperature, and in 7 to 8 days pure growths of the ciliate developed.

### Methods

With free-living, coprozoic and intestinal protozoa all work must be initiated with contaminated cultures. It is advantageous to inhibit the bacterial growth. The present writers obtained good results by using dilute media low in proteins.

Ordinary hay infusion was sometimes used, but a medium which more consistently gave excellent results was prepared as follows: Approximately 50 gm. of timothy hay was cut into fine pieces and 1000 cc. of hot water poured over it. This was infused in the refrigerator for 24 hours, filtered through cotton, and 5

per cent horse serum added to it. After autoclaving, it was cleared with 10 gm. of Merck's unscented, white talcum powder, filtered through paper and again autoclaved. This constituted the stock solution, 20 cc. of which was diluted with 100 cc. of tap water, distributed in 10 or 12 cc. amounts and autoclaved, when needed. The final solution usually gave a neutral reaction; but if not, it was adjusted to pH 7.0. It may be designated as "basic medium." When a solid medium was required, 1.5 to 2 per cent agar was added, and for fermentation tests 1 per cent of various carbohydrates.

A dilute nutrient bouillon, consisting of 20 cc. of standard bouillon to 100 cc. of tap water, was also found favorable to some protozoa. Yet another medium, known as "hay infusion horse blood agar," consisted of basic agar medium plus 10 per cent horse blood. Noguchi's semisolid *Leptospira* medium was employed in a modified form. This was prepared by adding 2 per cent agar to the basic medium, after which 100 cc. of the mixture was added to 900 cc. of tap water. The completed medium was distributed in 10 or 12 cc. amounts and autoclaved.

Vegetable media were frequently used to advantage. Potato, carrot, or mangle beet water was prepared by cutting out pieces (wt. 2 gm.) of the vegetables with a cork borer, distributing them in tubes containing 10 cc. of tap water and autoclaving. For media, 2 cc. of the vegetable water was added to 10 cc. of sterile tap water. Raw potatoes were also employed. They were first scrubbed with hot tap water, then partially dried by heat, and an area was washed thoroughly with 70 per cent alcohol and flamed until the surface was charred, after which cylinders were cut out with a sterile No. 5 cork borer and placed in sterile petri dishes to be cut into pieces  $\frac{1}{2}$  inch in length. One piece was placed in each of a number of sterile tubes, containing 12 cc. of sterile tap water.

Low temperature is an important factor in inhibiting the growth of many bacteria. Most of our initial protozoa cultures were held at room temperatures which fluctuated throughout the year between 22° to 29°C.

### *The Separation of Flagellates or Ciliates from One Another by Dilution*

Sometimes more than one species of protozoan developed from the original inoculum. When this occurred the following procedure was used.

Cultures of a mixture of species were inoculated into hay infusion or another medium and held at room temperature. After a few days, when a good mixed growth had been obtained, separation was undertaken. A series of drops of sterile water were placed on a glass slide, and a standard platinum loopful of the culture was added to the first of them and mixed. From it a loopful was carried over to the second drop of water, mixed, and a loopful from the second drop carried into the third drop. Such transfers were continued through six to eight drops or more. Each was examined microscopically with a low power objective until one was found which contained only a single protozoan cell. This drop was then drawn up into a fine pipette, inoculated into a medium and incubated at room temperature.

*Use of the "Migration Reaction"*

It was found that all of the protozoa here discussed were either positively or negatively geotropic, the majority being negatively so.

For the pipette procedure sterile glass tubes were employed, 14 or more inches long with a  $\frac{1}{4}$  inch bore and a fine tapering point. The larger ends were plugged with cotton and the tapering points rapidly flamed after removing the pipette from its sterile container. They were filled with sterile water to within about 2 inches of the top by applying suction through a rubber tube temporarily attached to the cotton-plugged end. About 2 cc. of the contaminated protozoan culture was sucked up into the pipette and formed a layer beneath the water. The tapering end was then sealed by heat. Care was taken throughout not to introduce air bubbles since they carried bacteria to the top of the water. A small sterile test tube was inverted over the cotton plugged end of the pipette and with sealed end down it was placed in a test tube rack (Fig. 1).

Many protozoa exhibited negative geotropism and usually after 5 or 10 minutes were found swimming near the surface of the water. With large forms the migration upwards could be observed with a hand lens, but with small species it was necessary to remove samples occasionally from the surface for microscopic search. In such cases it proved more convenient to invert small sterile test tubes over the open ends of the pipettes than to stopper them with cotton plugs. After  $\frac{1}{2}$  hour many protozoa appeared at the surface. By migrating through the long column of water they had washed themselves free of most other microorganisms. To completely free them a second washing was usually necessary. Sometimes the protozoa were left in the first pipette for from 14 to 18 hours. This longer sojourn often proved advantageous with forms that ingest bacteria or their spores since time was thus allowed for their evacuation.

The second washing was effected by filling another sterile pipette with sterile water up to about 2 inches from the top and then drawing up about 2 inches of fluid from the surface of the first pipette. Again the tapering end was sealed and the pipette placed in the rack. Protozoa usually came to the surface within a few minutes to  $\frac{1}{2}$  hour, depending upon the species. Following this second migration, media were ordinarily inoculated with a drop of the surface fluid. A third washing was occasionally necessary.

Tests showed that the migration to the top of the pipettes was a genuine geotropic reaction, not heliotropic, nor a positive response to oxygen. It occurred in pipettes held in the dark and in those sealed without any air space at the top. The protozoa did not float up for they were heavier than water and could be readily centrifuged to the bottom. The factors which effect geotropism of *Paramecium* were recently investigated by Dembowski (12).

Occasionally "V" shaped tubes (Fig. 2) instead of pipettes were used for the purification procedure. One arm of such a tube measured 12 cm. in length, with an inside diameter of 28 mm. The other arm measured 9 cm. in length, with an

inner diameter of 8 mm. This tube was sterilized and filled with 15 cc. of melted Noguchi's semisolid medium modified with the basic medium. After partial solidification of the medium, some material from contaminated cultures of the protozoa, 48 hours old, in basic medium, was inoculated into the "V" shaped tube. This was accomplished through a long fine capillary pipette introduced through the small arm into the large arm, the inoculum being placed in the bottom. Great care was taken to prevent the formation of air bubbles. The inoculated "V" tube was permitted to stand at room temperature for a length of time dependent upon the species of protozoan involved, and then samples from the surface of the medium in the large arm were taken. This method was found better for a few species than the pipette technique, because it often yielded 25 or more protozoa per 0.1 cc. of medium and a larger percentage of bacteriologically sterile cultures.

In concluding this section it may be noted that no protozoan culture after incubation was pronounced free from bacteria unless stained films, aerobic and anaerobic cultures on routine laboratory media, consistently proved negative.

### *Application of Methods*

#### *Trichoda pura*<sup>1</sup>

This ciliate ingests bacteria freely when in the contaminated state. It was obtained in ordinary hay infusion from stream water, together with *Euglena*, bacteria, and other microorganisms.

*Trichoda* was first separated from *Euglena* by the dilution method and then inoculated into the basic medium and incubated at room temperature for 48 hours or longer. When a good culture had been obtained, the ciliate was purified from contaminating bacteria by washing twice in pipettes. Each washing was effected in approximately  $\frac{1}{2}$  hour. Table I shows the results obtained after 4 days incubation in 10 tubes of basic medium inoculated with the surface migrants obtained from the second washing. The inoculated tubes were held 10 days without yielding any further results.

A pure culture of *Trichoda* was obtained in Tubes 4 and 8; bacteria developed in Tube 3, and the other tubes remained sterile. Bacterial sterility was checked culturally on standard media and by making stained films.

To test the efficiency of the two washings a pure *Trichoda* culture was purposely contaminated with a heavy suspension of a staphylococcus culture and incubated 48 hours at room temperature. The *Trichoda* were then washed twice and the surface migrants from each washing inoculated into 6 tubes of basic medium.

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<sup>1</sup> We are indebted to Dr. David Causey for the identification of this species.



Table II shows that at least two washings were necessary. It is also clear that *Trichoda* developed more rapidly when bacteria were present. However, once a bacteria-free culture had been initiated the protozoa developed well.

*Trichoda pura* when uncontaminated grows best in our basic medium, the reaction of which it changes in 2 weeks from pH 7.0 to pH 6.6. It also grows well in potato and carrot water. Moderate growth is obtained in ordinary hay infusion, standard bouillon, and whey (1.5 cc. whey to 8 cc. H<sub>2</sub>O). *Trichoda* does not grow on solid

TABLE I

Culture No.....	1	2	3	4	5	6	7	8	9	10
Result in 4 days.....	0*	0	C	+	0	0	0	+	0	0

\* 0 signifies sterility; C bacterial contamination; + pure culture of protozoa.

TABLE II

First washing						
Culture No.....	1	2	3	4	5	6
Result in 48 hrs.....	+C**	+C	+C	+C	+C	+C
Second washing						
Culture No.....	1	2	3	4	5	6
Result in 6 days.....	+	+	+	+	+	+

\*\* +C signifies protozoa contaminated with bacteria.

media, but good growths are obtained in the condensation fluid of standard and blood agar slants. The protozoan is an aerobe and on Noguchi's semisolid medium, modified with basic medium, growth occurs on the surface as a white cloud. This semisolid medium is used for the maintenance of stock cultures since they persist longer in it than in other media. It is advisable, however, to transplant once a month. No growth of *Trichoda* occurs in milk. Gelatin is completely liquefied in 12 days. No gas is produced in sugar solutions, but dextrose and mannose are fermented. Incubator temperatures kill

*Trichoda*. No true cysts have been found in any of the cultures, although round or resting stages appear to occur.

### *Undetermined Large Ciliate*

By inoculating some pond water into basic medium a large ciliate was obtained in 24 hours, which resembles some of the species of *Balantidium*. In contaminated cultures the ciliate, like *Trichoda*, ingests bacteria freely. Nevertheless it was easily purified.

A single pipette washing proved necessary, because migrants reached the top of the water column in 15 minutes, that is to say, long before bacteria. 0.1 cc. of this surface water yielded approximately 5 to 6 individuals. 40 per cent of all tubes of basic medium, inoculated with 0.1 cc. amounts of the water containing

TABLE III

Culture No.	Incubation time		
	24 hrs.	48 hrs.	72 hrs.
1	0	0	+
2	0	0	0
3	C	+C	+C
4	C	+C	+C
5	0	0	+
6	0	0	+
7	C	+C	+C
8	0	C	C

the migrants, gave bacteria-free cultures. The growth of this protozoan on other media was not tested. It develops only at room temperature.

### *Chilomonas paramecium*

This flagellate was obtained by inoculating hay infusion with pond water. It does not ingest bacteria for food.

The first pipette washing was effected in 30 minutes. The surface migrants in the second washing were scarce after 30 minutes, so the pipette was held for 20 hours, during which time the *Chilomonads* increased considerably at the surface, owing to migration and multiplication. A third washing was made from the second within 30 minutes. Eight tubes of basic media were each inoculated with one drop from the surface of each washing. The tubes were held for 72 hours and then discarded if no protozoa were observed microscopically. After 5 to 6 days

*Chilomonads* could be seen macroscopically. When observed they appeared as a delicate surface growth with fine flocculations which fell in straight lines to the bottom of the media when the tubes were disturbed.

Cultures from the first washing yielded bacterial growths in the 8 tubes within 24 hours, and *Chilomonas* also appeared within 48 hours. The same result was obtained from the second washing. The results of the third washing are given in Table III.

*Chilomonas* was obtained in pure culture in tubes 1, 5 and 6. It became visible 24 hours earlier in contaminated than in pure cultures.

*Chilomonas paramecium* when pure grows best in basic medium and in the modified semisolid medium. No growth was obtained in standard or dilute (1/5) bouillon, on standard or blood agar, or on hay infusion serum agar (solid basic medium). The microorganism did not ferment carbohydrates.\* No cysts were observed in any of the cultures. Incubator temperatures destroyed this species.

#### *Undetermined Flagellate of the Family Monadidae*

This microorganism does not ingest bacteria. The body is sometimes round but ordinarily pear-shaped, measuring 9 to 13  $\mu$  long by 6 to 11  $\mu$  wide. No axostyle or fibre is present. Division by binary fission occurs. It possesses four active flagella at anterior end, and forms cysts in old or unfavorable cultures. The monad was obtained by inoculating basic medium with pieces of comminuted wood from the galleries of the white ant, *Leucotermes flavipes*.

The flagellate can be purified in either of two ways, owing to the fact that a migration in both directions begins within the pipettes in about 10 minutes. One may wash as in the cases already described, or may fill the pipettes with sterile tap water and place about 0.1 cc. of a good mixed culture on top of the column. It is best to take advantage within 30 minutes of the downward migration of some of the protozoa. Thereafter a gradual reversal of the positive geotropism of some individuals occurs and all react negatively within 24 hours. If the positive geotropism is utilized, two washings are made, consuming about 30 minutes each. At the conclusion of the first the end of the pipette is cut off and a drop or two of water containing the migrants is introduced into the top of the water column in another pipette. At the conclusion of the second washing one or two drops of the water containing the bottom migrants are introduced into tubes of basic media.

Table IV gives the cultural results obtained after two experiments involving utilization of the positive geotropism after the second washing.

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\* Glucose, galactose, mannose, lactose, sucrose, maltose, mannite and raffinose.

When negative geotropism is employed for the purification, four longer washings are necessary, and it is advantageous to add about 0.5 cc. of the basic medium to the surface of the water. This appears to exert a stimulating effect. The first and second washings are permitted to stand approximately 48 hours, whereas the third and fourth are completed within 45 minutes to 1 hour.

TABLE IV

First experiment			
Culture No.	Incubation time		
	24 hrs.	48 hrs.	72 hrs.
1	0	0	+
2	0	0	0
3	0	0	0
4	0	C	C
5	0	+	+
6	0	+	+
7	0	0	0
8	0	0	C
9	0	0	C
10	0	0	0

Second experiment			
Culture No.	48 hrs.	72 hrs.	96 hrs.
1	0	0	+
2	0	0	0
3	0	+	+
4	0	+	+
5	0	+	+
6	0	+	+
7	0	+	+
8	0	+	+

Table V gives the cultural results, obtained after two experiments involving the use of the negative geotropic reaction, following the fourth washing. By comparing Tables IV and V it will be seen that both methods yielded good results; but the use of the positive geotropic reaction was preferable because it consumed less time.

The monad is destroyed by incubator temperatures. When pure, a delicate but good growth is obtained in basic medium within 5-6 days at room temperatures. A better growth appears if a piece of

fresh guinea pig kidney is placed in the bottom of the tube. Under these conditions a flocculent development occurs about an inch above the tissue. A poor growth occurs in ordinary hay infusion. No colonies are formed on basic medium agar, although development occurs in the condensation fluid. A good growth occurs in Noguchi's

TABLE V

First experiment			
Culture No.	Incubation time		
	48 hrs.	72 hrs.	96 hrs.
1	0	0	+
2	0	0	0
3	0	+	+
4	0	0	+
5	0	0	+
6	0	+	+
7	0	+	+
8	0	+	+
9	0	+	+
10	0	+	+
Second experiment			
1	0	0	C
2	0	0	0
3	0	+	+
4	0	0	+
5	0	C	C
6	0	+	+
7	0	+	+
8	0	0	+
9	0	+	+
10	0	0	0

modified semisolid medium. A moderate growth was obtained in potato, carrot and mangle beet water. No growth occurs in standard bouillon, or on standard nutrient agar, blood agar, or dextrose blood agar. The species does not produce gas nor does it ferment any of the carbohydrates used.<sup>2</sup>

<sup>2</sup> Glucose, galactose, mannose, lactose, sucrose, maltose, mannite, raffinose.

*Parypolytoma satura*

During the summer of 1928 this protozoan was obtained from the alimentary tract of adult house flies (*Musca domestica*). On one occasion the flagellate was found in 2 out of 20 flies, on another in 5 out of 30. At present the writers are unable to state whether the species is at times parasitic in house flies or constitutes a free-living form occasionally swallowed with the food. It is a flagellate which does not ingest bacteria and apparently forms no cysts in old or unfavorable cultures.

Basic medium was inoculated with a drop of a saline solution containing the alimentary tract contents in which were the flagellates. After 4 days a mixed culture of bacteria and protozoa was obtained. The flagellates were separated from the bacteria by permitting them to migrate once to the surface through a column of water. Every half hour a loopful from the surface was examined under the microscope. In about 2 hours 2 to 3 flagellates per field were counted at 100 diameters magnification. Small amounts of the fluid (0.1 cc.) were now taken from the surface of the water with a capillary pipette and 10 tubes each of basic medium inoculated with this amount. The tubes were incubated at room temperature for 5 to 6 days. After this time pure delicate cultures of *Parypolytoma* were obtained in 3 tubes out of 10, while 3 others remained sterile and 4 contained bacteria as well as protozoa.

The flagellate contaminated with bacteria grows very luxuriantly in the basic medium. When pure, however, it yields in 5 days a delicate culture in this medium with approximately 10,000 to 15,000 individuals per cubic centimeter. Basic medium to which 5 per cent laked horse blood has been added furnishes a good growth of the flagellates in 5 days. By the end of this time a fine flocculation has occurred throughout the medium with a small amount of sediment. In 10 days the medium clears with the production of a considerable sediment. The flagellates when contaminated with bacteria do not colonize on solid media. However, after purification and adaptation to artificial conditions colonies are formed. On horse blood agar slants minute dewdrop colonies develop in 3 days and a flocculent growth also appears on the surface of the condensation fluid. Noguchi's semisolid medium, modified with 10 per cent horse serum, yields a delicate growth on the surface in 5 days. In 10 days a diffuse growth occurs throughout this medium. No growth develops in hay infusion,

standard bouillon, or nutrient agar, although in the condensation fluid of the latter medium a few active and many rounded resting or degenerating forms are found. The protozoan does not ferment glucose, galactose, mannose, lactose, sucrose, maltose, mannite or raffinose.

Incubator temperatures inhibit growth, but exposure for short periods of time does not kill the flagellates. When held at temperatures of from 30° to 35°C. for 1 or 2 days, and then removed to temperatures ranging between 22° to 28°C., the protozoa begin again to grow.

TABLE VI

Culture No.	Migration time in minutes	No. of protozoa per 0.1 cc. of surface water	Incubation time in days					
			1	2	3	4	5	10
1	5	0	0	0	0	0	0	0
2	10	0	0	0	0	0	0	0
3	15	0	0	0	0	0	0	0
4	20	1	0	0	0	0	0	0
5	25	2	0	0	0	0	0	0
6	30	3	0	0	0	0	0	0
7	35	6	0	0	0	0	0	0
8	40	many	0	+C	+C	+C	+C	+C
9	50	many	0	+C	+C	+C	+C	+C
10	60	many	0	+C	+C	+C	+C	+C

#### *A New Flagellate from the Intestine of Lucilia caesar*

This parasitic flagellate is being described elsewhere<sup>3</sup> as a new member of the family *Monadidae*. In July, 1929, 6 out of 20, or 30 per cent of the adults of *Lucilia caesar* were found infected with it. It occurs in large numbers in the intestines of the flies and ingests bacteria freely. No cysts were observed in flies, nor later in cultures.

In the contaminated state a good growth of the flagellate was readily obtained in basic medium. The microorganism was repeatedly separated from bacteria by the pipette method, but when pure it did not grow in basic medium nor in many other media. When a contaminated culture was diluted and streaked on basic medium agar, the protozoan multiplied but did not colonize separately. Its individuals remained localized within and around bacterial colonies upon which they appeared dependent.

<sup>3</sup> Accepted for publication by the *Journal of Parasitology*.

The *Lucilia* flagellate was subjected to only one washing. Table VI shows that protozoa free from bacteria appeared at the surface of the water column within 20 minutes. Within 20 to 35 minutes sterile protozoa were introduced into basic medium, but no growth was obtained. Washings consuming from 40 to 60 minutes yielded contaminated protozoa cultures, because bacteria also reached the surface.

It proved possible to grow the flagellate in association with but one bacterium, namely, a Gram-negative coccus. Thus a "pure mixed culture" was obtained. When heavy agar growth suspensions of the bacterium, killed by heating at 60°C. for 30 minutes, were added to the basic medium containing some purified flagellates, cultures did not develop, a fact which led us erroneously to suppose that the flagellate will not grow in the absence of living bacteria.

It was thought that perhaps if a greater number of purified protozoa were obtained to begin with, those that died first might furnish the necessary factors for

TABLE VII

Culture No.	Incubation time in days					
	4	5	6	7	8	15
1	+	++	++	++	++	++
2	+	++	++	++	++	++
3	+	++	++	++	++	++
4	C	C	C	C	C	C
5	+	+	+	++	++	++

the growth of more vigorous individuals. For the purpose the "V" shaped tube, already described and figured (Fig. 2), was employed. The inoculated "V" tube, filled with semisolid medium, was permitted to stand at room temperature for from 12 to 15 hours. At the end of this time samples from the surface in the large arm were taken and frequently yielded 25 or more uncontaminated protozoa in each 0.1 cc. of medium. In other words, the protozoa reached the surface in the large arm before the bacteria did. The flagellate, however, at first refused to grow after the purification, although various media were tried, with the addition of heat-killed bacteria or yeast and autolyzed yeast. Bacterial filtrates added to the culture media did not exert any stimulating effect. Control tubes, to which living bacteria or yeast cells were added, produced luxuriant growths of the flagellate.

The writers had previously found that the purified protozoan would not develop on autoclaved potato. However, as a matter of routine, some tubes of raw potato under tap water, described in the section on media, yielded a good pure culture of the flagellate in 5 days. This experiment was repeated, and Table VII gives the results obtained by inoculating 5 tubes of raw potato water medium with the flagellate migrants from the surface of the large arm of a "V" tube.



Cultures obtained in this manner were never luxuriant as under conditions of contamination. The pure growth was very delicate. To date the organism has been under cultivation approximately 5 months. In the raw potato water the flagellate grew at both incubator and room temperatures (30–35° and 22–28°C. respectively).

### *Spirillum undulans*

This widespread microorganism was obtained from some stream water sediment. It will be included here, since it was purified by the technical methods described.

The *Spirillum* as obtained was mixed with protozoa. A pipette was filled with 8 cc. of sterile tap water, and 2 cc. of the stream water with the sediment was placed below. The tube was permitted to stand all night, during which time the protozoa migrated to the surface, whereas the *Spirilla* remained at the bottom of the pipette. The end of the pipette was now cut off and the sediment inoculated into some chocolate horse blood. Within 5 days at room temperature heavy growths of *Spirilla* and other bacteria were obtained.

The *Spirillum* was purified as follows: A platinum loopful of the scum from the surface of the chocolate blood cultures was placed on the surface of sterile tap water in the large arm of a "V" shaped tube. This was done without shaking the tube which was permitted to stand for 1 hour at room temperature, when 0.5 cc. amounts from the surface of the small arm were withdrawn, and chocolate horse blood medium inoculated. At the end of 11 to 14 days, at room temperature, pure growths of the *Spirilla* appeared. These pure growths, however, were not as luxuriant as the contaminated cultures.

A contradiction seems to be involved in this work with the *Spirillum*. During its separation from protozoa, the microorganism was after the first 16 hours found at the bottom; but it migrated to the surface of the small arm of the "V" tube (1 hour) during the later purification. The results mean merely that reversals of tropic reactions occur.

Basic medium, to which sterile rabbit kidney was added, furnished a better medium for the purified *Spirilla*. After 48 hours, a delicate haze was observed in this medium, beginning at the tissue and extending almost to the top of the liquid. After a few days a rather dense disk was formed at the surface. The *Spirillum* withstood incubation (30–35°C.), but lived longer at room (22–28°C.) temperatures; and it survived longer when the medium was covered with a layer of sterile paraffin oil. The pure microorganism also grew well in Noguchi's *Leptospira* medium or in plain hay infusion, provided sterile kidney was added. Indeed, sterile tap water with rabbit or guinea pig kidney answered very well. A growth was not obtained in standard laboratory media.

*Paramecium caudatum*

This ciliate, which ingests bacteria and yeasts freely, was readily obtained free from other forms, but up to the present has not reproduced itself in the absence of living microorganisms. The findings constitute a verification of the experiments of Hargitt and Fray (13), Phillips (14), and Parpart (11). The best purification possible by the present writers was obtained by associating *Paramecium* with a bacterium or with a yeast, *Saccharomyces cerevisiae*.

The procedure used for obtaining vigorous "mixed pure cultures" of *Paramecium* follows: The contaminated ciliate was inoculated into the basic medium to which some pure living yeast cells had been added. The yeast stimulated the protozoa, and within 5 to 6 days a luxuriant culture of *Paramecium* was obtained. The cul-

TABLE VIII

Culture No.	Time in days when growth appeared, with degree of growth														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	—***	—	—	±	±	±	±	+	++	+++	++++	++++	++++	++++	++++
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	±	±	±	±	+	+	++	++	++	+++
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

\*\*\* — signifies no growth; ± doubtful; + weak growth; ++ fair growth; +++ good growth; ++++ luxuriant growth.

ture was then centrifuged for about 2 minutes at low speed and the upper liquid discarded quickly, before the *Paramecia* rose to the surface. The sediment was washed and centrifuged four times with sterile tap water. As much water as possible was removed from the last washing. The final sediment consisted mainly of *Paramecia* and yeast cells.

About 10 to 15 cc. of sterile tap water was now drawn up into one of the long pipettes, followed by approximately 0.25 cc. of the sediment. The pipette was permitted to stand at room temperature for about 18 to 24 hours, at the end of which time nearly all of the *Paramecia* migrated to the surface of the water and evacuated the remains of most of the ingested microorganisms. The surface migrants were washed a second time. The pipette was carefully observed with a hand lens, and it was seen that many of the ciliates ascended in about 20 to 30 minutes. At this time 0.1 cc. amounts from the surface of the water column yielded about 12 *Paramecia*. Basic and other media were inoculated with 0.1 cc. amounts of the

fluid. On incubation such media were found entirely free from contaminating microorganisms.

If the media containing *Paramecium* were inoculated with a pure yeast culture, a growth of the ciliate was obtained in some of the tubes in from 8 to 10 days at room temperature. These cultures became sturdy in from 14 to 15 days, as shown in Table VIII.

After the cultures with yeast had been initiated, reinoculations of aliquot amounts into basic medium containing living yeast yielded growth more rapidly, as shown in Table IX.

The experiment was performed of adding 1 cc. of a heavy suspension of yeast cells killed by heat (60°C., 1 hour) to five tubes of basic medium inoculated with purified *Paramecia*. No growth of the ciliate resulted, whereas in the control tube containing living yeast it took place as usual.

In another experiment, autolyzed yeast was added to five tubes of basic medium

TABLE IX

Culture No.	Time in days when growth appeared, with degree of growth				
	1	2	3	4	5
1	±	±	+	+++	++++
2	±	±	+	+++	+++
3	±	±	+	+++	+++
4	±	±	+	++	++
5	±	±	±	+	++

inoculated with purified *Paramecia*. The yeast cells had been autolyzed by agitating them in chloroform for 15 days until a gummy mass was obtained. The chloroform was then evaporated and the autolyzed yeast suspended in basic medium. No growth took place in the tubes of medium thus prepared, whereas in the control containing living yeast it occurred as usual.

From the foregoing experiments it appears that *Paramecium caudatum* requires living microorganisms. It is possible though that the required accessory food factors have merely not yet been found.

When *Paramecia* were grown in the presence of yeast cells the media remained clear. The yeast largely adhered to the glass, but the protozoa were active everywhere in the tubes. The colonies of yeasts on the glass attracted large numbers of *Paramecia*, which ate out holes within the colonies, and finally devoured them completely. Mangle beet water proved an excellent medium for *Paramecia*, probably because it supports a luxuriant yeast growth.

*Euglena proxima*

This flagellate was found after inoculating some hay infusion with stream water. Professor Gary N. Calkins informed us that it corresponds more nearly to *Euglena proxima* of Dangeard than to any other, and he regarded it as a variety of this species. It does not ingest bacteria.

TABLE X

Length of exposure	Concentration of potassium dichromate	Time in days when growth appeared									
		1	2	3	4	5	6	7	8	9	10
<i>min.</i>	<i>per cent</i>										
5	5.0	0	0	0	0	0	0	0	0	0	0
	2.50	0	0	0	0	C	C	C	C	C	C
	1.25	0	0	C	C	C	C	C	C	C	C
	0.63	0	0	C	C	C	C	C	C	C	C
	0.31	0	0	C	C	C	C	C	C	C	C
10	5.0	0	0	0	0	0	0	0	0	0	0
	2.50	0	0	0	0	0	0	0	0	0	0
	1.25	0	0	C	C	C	C	C	C	C	C
	0.63	0	0	C	C	C	C	C	C	C	C
	0.31	0	0	C	C	C	C	C	C	C	C
15	5.0	0	0	0	0	0	0	0	0	0	0
	2.50	0	0	0	0	0	0	0	0	0	0
	1.25	0	0	C	C	C	C	C	C	C	C
	0.63	0	0	C	C	C	C	C	C	C	C
	0.31	0	0	C	C	C	C	C	C	C	C
30	5.0	0	0	0	0	0	0	0	0	0	0
	2.50	0	0	0	0	0	0	0	0	0	0
	1.25	0	0	0	0	+	+	+	+	+	+
	0.63	0	0	0	C	C	C	C	C	C	C
	0.31	0	0	C	C	C	C	C	C	C	C

The writers were unable to free *Euglena* from bacteria by any method involving migration or plating, so a technique of chemical sterilization was devised. It proved very efficient, especially when used on vigorous cultures 48 hours or more old which contained round or encysted stages.

A standard platinum loop was charged 5 times with a contaminated *Euglena* culture, and each charge was deposited in 5 separate places on a glass slide. A charge of the same loop filled with a 10 per cent potassium dichromate solution was then deposited in the first drop of the contaminated *Euglena* culture and stirred.

This theoretically reduced the potassium dichromate to 5 per cent. A charge of this mixture was carried over with the loop to the second drop of the culture. This was again stirred, reducing the dichromate to 2.5 per cent. The procedure was repeated until the fifth drop gave a dichromate concentration of approximately 0.31 per cent. The culture was now exposed to the dichromate concentrations for varying lengths of time in a moist chamber. After each exposure a loop from each of the 5 drops was inoculated into tubes of sterile hay infusion and incubated at room temperature.

TABLE XI

Concentration of potassium dichromate (15 min. exposures)	Time in days when growth appeared									
	1	2	3	4	5	6	7	8	9	10
<i>per cent</i>										
5.0	0	0	0	0	0	0	0	0	0	0
2.50	0	0	0	0	0	0	0	0	0	0
1.25	0	0	0	0	+	+	+	+	+	+
0.63	0	0	C	C	C	C	C	C	C	C
0.31	0	0	C	C	C	C	C	C	C	C
5.0	0	0	0	0	0	0	0	0	0	0
2.50	0	0	0	0	0	+	+	+	+	+
1.25	0	0	0	C	C	C	C	C	C	C
0.63	0	0	0	C	C	C	C	C	C	C
0.31	0	0	C	C	C	C	C	C	C	C
5.0	0	0	0	0	0	0	0	0	0	0
2.50	0	0	C	C	C	C	C	C	C	C
1.25	0	0	C	C	C	C	C	C	C	C
0.63	0	0	C	C	C	C	C	C	C	C
0.31	0	0	C	C	C	C	C	C	C	C

Tables X and XI give the results of two experiments in which contaminated *Euglena* cultures were exposed for four time intervals to five concentrations of potassium dichromate. Three pure *Euglena* cultures were obtained within 5 to 6 days after exposure of from 15 to 30 minutes to 1.25 to 2.50 per cent concentrations of the dichromate.

When *Euglena proxima* was grown in the pure state in the presence of light it produced chlorophyl in greater abundance than was the case with contaminated cultures. It grew best in basic medium, potato water, and in diluted standard bouillon (1/5), but grew well also in hay infusion, and on the surface of basic and standard agar

which showed after 2 to 3 weeks green colonies of 1 mm. diameter. Although *Euglena* colonized in solid media, it could not be purified by taking advantage of this fact. The flagellate grew poorly in standard bouillon, carrot water, and on ordinary hay infusion agar. The microorganism grew throughout basic agar stabs and shakes, and in modified Noguchi's semisolid medium, behaving like a facultative anaerobe. In litmus milk a soft coagulum was formed without the production of acid. Gelatin was not liquified, although a good growth occurred. In media which contained sugars and starches, no gas was produced, and dextrose, the only carbohydrate fermented, gave a final pH reading of 6.2. Incubator temperatures inhibited the growth of *Euglena*.

#### SUMMARY

Some media are described which inhibit bacterial growth, but are favorable to protozoan development.

A purification technique, which takes advantage of geotropic responses, was devised and used successfully with 7 species of protozoa, including flagellates and ciliates. The method was also used with a *Spirillum*. For one flagellate which could not be purified in this manner, a procedure involving chemical sterilization was employed. *Paramecium caudatum* was purified, but failed to develop subsequently in the absence of living microorganisms. Four of the protozoa which were purified ingest other microorganisms normally.

The work shows that purified protozoa grow well under proper conditions, and then they can be studied culturally and biologically, like bacteria.

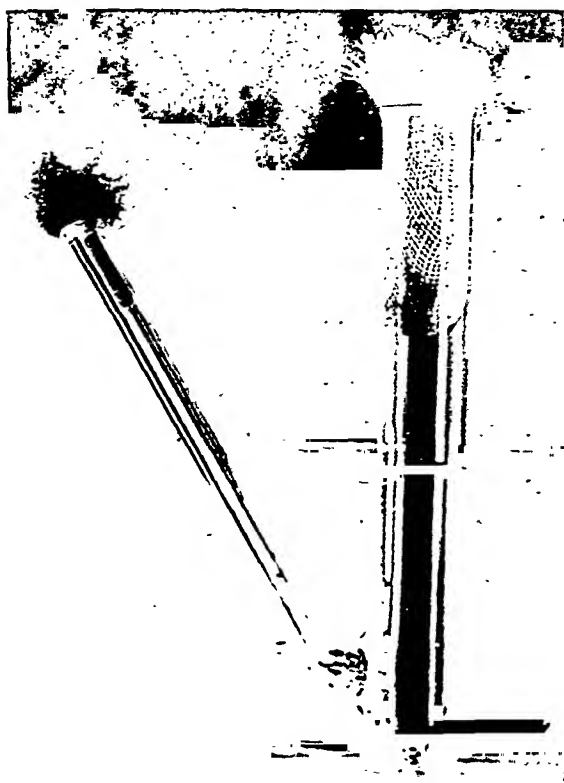
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#### EXPLANATION OF PLATE 13

- FIG. 1. "Set up" used in migration experiments with liquid medium.
- FIG. 2. "V" tube used in migration experiments with semisolid medium.



(Glaser and Cori: Pure culture of protozoa)





# THE GRADIENT OF VASCULAR PERMEABILITY\*

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PLATES 14 TO 17

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The main purpose of this paper and of others which follow is to bring proof of the existence in certain situations of a gradient of permeability along the capillaries which makes for the equalization of opportunity within the tissues served by them. Means are described whereby this equalization is achieved in different fashion elsewhere in the body, and evidence is brought forward of a significant permeability of the arterioles and venules.

## *The Distribution of Vital Stains within Skeletal Muscle*

The sheet muscles of guinea pigs and young rabbits are so transparent that the distribution of innocuous dyes can readily be followed during life.

The smallest vessels of voluntary muscle are arranged almost diagrammatically (1), with arterioles and venules disposed alternately in the same plane, at approximate right angles to the muscle fibres (Figs. 1, 2, and 3; Schema I, A). The numerous, parallel capillaries which bridge the gaps between are, in the rabbit, often more than 1 mm. in length. Certain of them pass the nearest venule and course to that next beyond. All lie next the fibres which they nourish and adjoining ones are linked by occasional cross connections; but the existence of these does not significantly affect the rule that the blood leaving an arteriole finds its way back to the large vessels by way of the next venule.

To witness the distribution of vital dyes a window can be inserted over the external oblique, a "white" muscle. The rabbit is etherized after a fast of 18 to 24 hours, laid—not stretched—on its back, an oval piece is snipped with scissors

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\* Studies of Tissue Maintenance. IV.

from the shaved skin of the side of the belly, the subcutaneous layer is parted, and a plate of clear, dry mica,  $\frac{1}{2}$  to  $1\frac{1}{2}$  cm. in greatest diameter, is rapidly inserted over the muscle. It should be devoid of scratches, thin enough to be pliable, and of a size to fit snugly beneath the opening. Abnormal tensions and pressures must be avoided. In rabbits of 700 to 1500 gm. the fascia is glassy and so loose that the window set upon it can be pushed here and there to obtain a view of regions not directly exposed. In old individuals it is somewhat opaque, not infrequently contains fat, is fixed to a murky muscle; and more than one window must frequently be inserted to bring to view a field showing arteries and veins in the characteristic relation.

Brilliant pictures are obtained by underlaying the muscle with a curved strip of white celluloid inserted into the peritoneal cavity through a slit in the mid-line. But this complicating procedure is for special occasion.

As soon as the window has been placed the dye is injected into a vein while the muscle is watched through a binocular dissecting microscope, with the animal upon a warmed platform that can be tilted. The light from a Leitz carbon arc lamp, cooled by filtration through Magnus' fluid (2), is reflected upon the field by a plane mirror. The transverse venules appear broad and stumpy, the arterioles thread-like, and often indistinctly visible until the blood is stained.

To procure specimens at any special stage of the staining, the carotids are severed for exsanguination, the skin and cutaneous muscle are dissected away together, the oblique is cut through on three sides of a square (the fourth being toward the back), the free edge is seized with a clip of the sort used to hold photographic films while drying, and by gentle traction and dissection the piece of muscle is separated from the underlying tissue, everted upon a glass plate, covered with another, and cut loose. The location of the stain is best seen when the specimen is placed upon a white surface and viewed on its inner side; for the vessels mostly ramify near this side. The cutaneous muscle, internal oblique and transversalis also yield instructive pictures, while the serratus magnus, gracilis, adductors and pectorals provide corroboratory data.

The tissue of guinea pig muscle is so thin and clear that high magnifications can be used; but the external obliques of the cat and rat are ruddy and relatively opaque; and the abdominal wall of the mouse is so thin that its individual layers cannot easily be separated. In all these animals, however, the distribution of the dye takes the same course.

The staining is most easily followed with dyes which pass very slowly through the vascular walls. The findings with Chicago blue 6B (No. 518, Colour Index (3)) are typical.

Chicago blue 6B (General Dyestuff Corporation) has a molecular weight of 992 and is so slightly diffusible that dialysis can be utilized to purify the commercial preparation. A saturated watery solution of it is placed in the receptacle made

by clamping together the corners of a square of "diphtheria parchment paper" (Reeve Angel and Company), and this is immersed for 4 days, first in running tap water, then in distilled. Thymol must be added to prevent bacterial growth. It is removed later from the purified and dried dye by prolonged shaking with absolute alcohol. As thus prepared Chicago blue dissolves readily, an 8 per cent solution in water being isotonic with blood and having a pH of 8.3.\* The intravenous injection at body temperature of 5 cc. for each 1600 gm. of rabbit causes no symptoms in unanesthetized animals, when it is given in the course of 4 minutes; but the kymograph discloses in etherized ones a slight, almost momentary drop in blood pressure. A considerable interval elapses before the dye begins to escape from the vessels.

Following the injection the skin becomes only light blue; but it gradually darkens during the next 2 hours, while the muscle, lighter to begin with, becomes darker still. The external oblique watched through the mica shows a swift bluish shadowing as the stain reaches the viscera beneath, and a few seconds afterwards its arterioles and venules stand out in blackish blue to their least ramifications. The region between them now has an indistinct, azure striation owing to dye in the capillaries. These can be readily made out with the high power. The extravascular tissue itself is unstained.

The period before Chicago blue begins to leave the vessels varies with the age and state of the animal. In rabbits of 1600 gm. under light ether a gradually intensifying blue mist appears after 5 to 15 minutes, in the region served by the last third of each set of capillaries connecting transverse arteriole with transverse venule. With a low magnification the arterioles are easily recognizable in the midst of completely unstained tissue, whereas each of the venules alternating with them is surrounded by a blue cloud, or plume, with fading margins (Fig. 9). The result viewed in the gross is a fern-like marking of the external oblique (Fig. 8). With a watchmaker's eye-piece (magnification  $\times 1\frac{1}{2}$ ) one can see that the center of each blue plume is separated from the next by  $1\frac{1}{2}$  to 2 mm., by twice the gap between arteriole and venule that is to say; but there are some larger unstained spaces through the center of each of which a solitary arteriole courses. Here the distance to the nearest collecting venule is often  $1\frac{1}{2}$  mm. at the least. In long muscles,—gracilis, pectorals, and quadratus lumborum,—zigzag bars of color, suggestive of the markings upon a mackerel, lie transverse to the fibres (Fig. 11), as mentioned in a previous paper (4).

What is the cause for this barred coloration? It does not occur until after the dye has circulated for some minutes; and it persists when

\* The hydrogen ion concentration of the various dyes was determined with the glass electrode by Dr. Dole or by Dr. Mirsky, to both of whom we feel greatly indebted. The figures on pH and tonicity may hold only for the dye specimens with which we have dealt.

the vessels are washed out with salt solution. Evidently the stain has escaped into the tissues. This cannot have resulted from the injury of exposure, for it has happened everywhere through the muscle. Nor is it due to the ether. The typical barring comes about relatively soon in unanesthetized animals (rabbit, mouse), being found when they are decapitated at a blow. Local decolorization will not account for it, since the dye retains its character in the organism, being gradually stored in cells with result that the tissues appear dark blue even after many weeks. However, the amount of Chicago blue we have employed causes the blood to become completely incoagulable for some hours, and though producing no immediate symptoms may prove fatal after 4 or 5 days. Some of the animals succumbing had marked ascites, a development which suggests that the dye may injure the endothelium selectively, with the coloration in the neighborhood of the least venules as result.

It has seemed better to broaden our observations with the aid of other poorly diffusible dyes than to study the effects of a single one intensively. Pure trypan red, trypan blue, and trypan violet (Gruebler) have proved suitable for the purpose. Though so like in name these differ greatly in constitution; but all have large molecules and escape from the vessels slowly. All give rise to bars of color situated like those of Chicago blue in the region traversed by the last third of the muscle capillaries. Each bar intensifies toward its middle where lies a transverse venule.

A 4 per cent solution of trypan red (No. 438, Colour Index) is isotonic with the blood and has a pH of 9.21. Added in a 1 to 20 proportion to blood at pH 7.44 under paraffin oil it causes no change in reaction perceptible with the glass electrode. To effect a sharp staining  $2\frac{1}{2}$  cc. must be injected for 400 gm. of guinea pig. The dye leaves the vessels nearly as slowly as Chicago blue, so its relation to the small vessels can be studied at leisure.

Trypan violet (5) is isotonic in 3.75 per cent solution and has a pH of 8.67. We have injected 1.25 cc. for 400 gm. of guinea pig. It passes from the vessels *in vivo* at approximately the rate of trypan red and gives especially beautiful color pictures.

Trypan blue (No. 477, Colour Index) has been used merely in corroboration, as an isotonic solution (6 per cent, pH 8.92) in the same amount per kilo as trypan red. Like the latter it does not notably alter the reaction of blood when added in 1 to 20 proportion. For reasons as yet undetermined some dye specimens fail to stain intensely.

The possible influence of toxicity, staining affinities or pharmacological action to cause the barred staining with the dyes thus far mentioned can be dismissed from account. For not only do they all yield essentially the same findings as Chicago blue, but this has proved true as well of two highly diffusible, innocuous, dyes, namely patent blue V and brom phenol blue (6).

The diffusibility of the dyes has been determined by the method of Northrop and Anson (7). The following table shows the relative rates through water and gelatin, respectively. The two columns of figures are independent of each other.

Dye	Water	Gelatin
Chicago blue 6B.....	1.0	—
Trypan blue.....	1.17	0.74
Trypan red.....	1.34	0.86
Trypan violet.....	1.70	1.09
<i>Brom phenol blue</i> .....	3.2	3.2
Patent blue V.....	6.2	6.3
Dextrose.....	7.1	20.3
Phenol red.....	7.6	7.8
Urea.....	12.4	41.7

For the determinations in the second column the porous disc was filled with 8 per cent isoelectric gelatin. The amounts passing at 0°C. in successive periods of 30 minutes in the case of water, and 6 hours in that of gelatin, were quantitated against standard solutions in a colorimeter.

The speed with which brom phenol blue diffuses has been taken as the standard for each tabulation. One sees that in water it spreads 3.2 times as fast as Chicago blue 6B. The latter dye did not pass through gelatin in a measurable quantity in 6 hours. Dextrose spreads through water only a little more rapidly than patent blue V but through gelatin more than three times as quickly.

Patent blue V passes out into the muscle everywhere at once and special means are needed to demonstrate the barring when it is given in large quantity. Either the distribution must be slowed in some way or else checked at an early period. We have resorted to both procedures.

Patent blue V has been utilized in our previous work on tissue maintenance. Three cc. of an isotonic solution (8 per cent, pH 6.0) will color deeply a 1600 gm. rabbit, and even when injected very rapidly it produces no untoward effects. When it is given in the course of a minute the external oblique, watched through a

window, seems to turn greenish blue throughout, as if drenched; and drenched indeed it has been from the capillaries throughout their length. There is not the least indication of a barring with color then, or when the muscle is removed 3 minutes after the injection. But if the dye is placed abruptly in circulation and the carotids are cut the moment it is seen to color the blood in the least venules of the muscle, most informative specimens are got. Superimposed upon the diffuse coloration in far deeper blue is the familiar barring. The bars have as axes the collecting venules, but are broader than those caused by the poorly diffusible trypan dyes and by Chicago blue. They occupy about half the tissue between transverse venule and arteriole, fading off in the direction of the latter.

The suffusion is so rapid and deep as in many instances to obscure the barring which becomes plainly visible only when the sheet of muscle is stripped back. Fortunately the conditions in animals depleted by bleeding favor observations *in vivo*. By three or four large hemorrhages from a carotid cannula, at intervals of 5 to 10 minutes, the blood pressure is lowered to about 30 mm. Hg; a window is inserted as usual into the abdominal wall; and the stain thrown forthwith into circulation.

The veins are still prominent in the muscle of the bled animal but very few arterioles can be seen. The generality stand forth as the stain reaches them, which may not happen for nearly a minute; and 3 further minutes may elapse before the color of the venous blood notably alters. One perceives a dark line of blue, as if from an unseen pencil, glide smoothly along the arteriole and trace to the tip each least twig of an arborization only guessed at before. Then rapidly a mist of blue forms about the arterial twigs while the rest of the muscle is still without color (Schema I, B). Already the dye is escaping. The localized mist, which in the gross appears as a blue barring of the tissue about the arterioles, does not spread but instead is lost after some seconds in an even, general staining of the muscle beyond; and upon this as a background a new and broad pattern asserts itself secondarily, namely that of the familiar barring about the transverse venules (Schema I, B). During the few minutes while this develops by a passage of dye out of the slowed stream but little reaches the venous blood. Rapidly the diffuse staining between the bars grows deeper and soon they are lost in it. When they are most intense the picture observed *in vivo* is like that in a normal animal killed at once after injection. The secondary barrings are spaced at the same distance as those of Chicago blue, which could not be the case unless the early, arterial, barrings had completely disappeared.

Though patent blue V stains the tissue all along the muscle capillaries, it does so with increasing intensity as the transverse venules are approached (Schema I, B). In the experiments just described the bars of color were broader than those of Chicago blue or of the trypan stains. When the circulation has been slowed by bleedings the tissue first met is first stained from the feeble current of blood, as does not happen

when the pressure is normal and the stream swift; and in consequence a blue barring occurs about the arterioles (Schema I, B). But the law of first come first served in the depleted animal holds for a brief period only. The entire region traversed by the capillaries undergoes a rapid, even suffusion with color, and upon this is superimposed secondarily the barring about the venules.

The conditions are such as should lead, one would think, to an especially pronounced spread of dye from the first portion of the capillaries; for the dye reaches this portion first, is most abundant there, and there the blood is under most pressure. It loses so much dye in passing through the muscle that the venous blood appears dirty brown, or green at most, while the bars are developing, never a sharp blue like that of the arterioles. The greatest staining takes place precisely where the known conditions seem most unfavorable thereto.

Brom phenol blue yields corroboratory results.

Etherized rabbits receiving intravenously 5 cc. of an isotonic solution of brom phenol blue (4 per cent, pH 7.24) in the course of 7 to 10 seconds show a gradual, diffuse staining of the external oblique muscle, and a superimposed pronounced barring round about the transverse venules, which endures for 2 minutes or more. The bars extend with diminishing intensity nearly half way to the arterioles. In animals with the local circulation slowed by bleeding no staining like that with patent blue V takes place about the distributing arterioles before the dye has gone further, but on the other hand the diffuse coloration is so slight as to render the venous barring especially vivid (Schema II, D). As with patent blue V the staining is greatest where the blood is poorest in dye. While the bars are developing the blood in the collecting venules appears but little stained, whereas that in the arteries is intensely blue.

The findings (Schema II, D) are precisely what one would expect with a coloring matter greatly more diffusible than Chicago blue but considerably less so than patent blue V, if one assume that the same general laws govern the distribution of all three dyes.

### *The Gradient of Distribution*

Why were the muscles so singularly barred with color? Their fibres were little if at all stained, and though the interfascicular connective tissue became gradually and diffusely blue, neither it nor the contents of the vessels was responsible for the markings. These were traceable



to extravascular dye lying between the fibres of the individual muscle bundles in the regions supplied from the further portion of the parallel capillaries (Fig. 10). There are no interstitial structures in this situation which stain especially well and which recur with each set of the little vessels serving the fibres. The inequalities of hue were limited to the period when the dyes were first being distributed and hence must have resulted from local differences in the ease with which these passed from the blood, differences so great that most of the coloring matter got out precisely where the known conditions appeared least favorable.

As already stated, the dye of greatest diffusibility, patent blue V, can be seen in bled animals to pass into the tissue about the terminal arterioles before the slow stream can carry it further; but soon there occurs a blueing along the entire capillary way. One must conclude that the little vessels are permeable to this dye throughout their length. And it escapes from some larger vessels as well. In animals killed within a few seconds after injection one often finds a narrow zone of blue next the arteries from which the transverse arterioles are given off, vessels which can at this time be absolutely identified because the blood within the venules is uncolored as yet. Later, as the tissue generally becomes suffused with blue, the zone can no longer be discerned, but soon an intense, localized coloration develops next the veins which receive the transverse venules. These vessels and the arteries about which staining occurs are close to the limit of visibility for the unaided eye, and injections with gelatin-carmines prove them to be devoid of *vasa vasorum*. So rapid is the spread of the dye in tissue manipulated post mortem that reliable photographs of the color phenomena have not been obtainable.

With brom phenol blue (Schema II, D) no staining takes place by direct passage through the wall of arterioles, but after some minutes a blue zone develops next those veins receiving blood from the transverse venules. The broad blue bars which appear early about the latter vessels do not spread and disappear by merging with one another, but, like those of patent blue V, remain unchanged while a more gradual, even staining takes place elsewhere along the capillary; and as this staining intensifies their margins are lost in it, so that they appear to be narrower, and soon they are no longer to be recognized. It is certain that brom phenol blue, while escaping most readily near the venules, emerges everywhere along the capillaries.

The passage into the tissues of Chicago blue and the trypan stains is so slow that in animals with thin, nearly transparent, abdominal musculature (guinea pigs, young rabbits, kittens) one can tell precisely where dye first leaves the vessels (Schema II, C). It is where the capillaries enter the transverse venules directly, or unite near them to form tiny radicles (Figs. 9 and 10). Here a mist of blue, red or violet forms and envelops the transverse trunk in color. The outline of each

bar of mist is step-like because the dye extends further back along some capillaries than along others. Not infrequently these enter one side only of the venous trunk, and the staining is then confined to this side (Fig. 10). None occurs about the venous trunk beyond the region where capillaries enter it (Fig. 10), and none about the larger vein into which it gives, nor about any of the arterioles.

Instead of spreading and merging with one another the colored bars retain their size while an even staining gradually takes place between them. Evidently the capillaries are somewhat permeable throughout their length even to the most poorly diffusible of the dyes we have used. As the general staining intensifies the boundaries of the bands disappear in it, and at last they are totally obscured. In young etherized rabbits injected with Chicago blue this may take more than 4 hours.

These facts prove that the dyes pass out all along the capillaries, but most readily in the region where they unite into venules. From their proximal portion Chicago blue and the trypan dyes escape very slowly. Brom phenol blue and patent blue V pass out of the capillaries everywhere, though with special ease at their end; and the small venules are permeable to them as well. Patent blue V penetrates even the wall of small arterioles, staining the tissue next them.

The progressive increase in intensity of the staining as the venule is approached suggests the existence of a gradient of distribution along the capillaries. But an alternative explanation is possible, namely that a secondary dispersion occurs of dye escaping only at the veno-capillary junction or from the least venules. In such event one should see the dye emerge like smoke from a leaky stovepipe, and spread backwards in the direction of the arterioles. The phenomenon could not be overlooked with patent blue V or brom phenol blue which give rise quickly to intense, broad bars. It never occurs. Furthermore the mist of Chicago blue remains of the same dimensions for hours, proving that secondary spread of the dye through the tissues is extremely slow. True, extravascular color is first noted where the capillaries enter the venules; but it does not spread thence. The bar of stain materializes throughout its eventual situation as a mist of graded intensity from center to margin; and when first perceptible it has nearly its eventual breadth, enlarging later only to the extent that might be expected from increased visibility. One is forced to conclude that some gradient affecting the distribution of vital dyes exists along the further portion of the capillaries.

### *Factors Influencing the Gradient*

The gradient is but little affected by drastic circulatory changes.

Ordinary muscular activity does not essentially disturb it. The external oblique and leg muscles of a rabbit injected with Chicago blue 6B, which wandered about the room for a few minutes before being killed, and struggled when picked up, showed the characteristic picture. So too with an animal that had repeated convulsions owing to air embolism. If the external oblique of etherized rabbits is directly stimulated to rapid contraction by 60 to 120 induction shocks per minute diffuse staining takes place; but the conditions have little relation to those of life.

Often plethora was produced incidentally, as when the blood volume was increased one-seventh by the injection of isotonic trypan red solution. In some tests the animal was bled nearly to exsanguination, the systemic blood pressure being reduced to the tolerable lower limit before the dye was injected, with result that the arterioles were contracted almost to invisibility and the stained blood entering the capillaries could be seen barely to creep along them, so slight was the force behind it. None of these changes essentially altered the distribution of the stains. In certain instances the nerves to a hind leg were cut just before the dye injection, to increase the circulation. The gradient disclosed with Chicago blue 6B proved similar to that in the control limb, the mackerel barrings being identical in extent.

In tests previously reported in another connection (4), not only were the nerves cut but the animals were bled prior to injection. Staining proved diffuse in the paralyzed legs whereas barring took place in the normal ones. But highly diffusible dyes were used—patent blue V, brom phenol blue—and the animals were killed so late that diffuse staining was to have been expected wherever vasoconstriction in compensation for the diminished blood bulk had been prevented by nerve severance.

A shock-like state was produced in several etherized cats and rabbits prior to the injection of stain, by traumatizing the muscles of a hind leg, after Cannon's method (8). The eventual weight of the limb as compared with its fellow showed that an enormous extravasation had taken place into it during the period while the blood pressure was falling; and much if not all of the fall must be attributed to reduction in the blood volume. The uninjured muscles where colored at all were characteristically barred (Fig. 12). So too were they in animals depleted with hypertonic solutions given by mouth (9) as also when the muscle circulation was cut down during the period of staining by the slow intravenous injection of epinephrine or pituitrin (10), a procedure which enormously raised the carotid blood pressure.

When large amounts of the dyes are given, as in the experiments thus far detailed, the width of the colored bars varies directly, like the rapidity of the staining, with the diffusibility of the coloring matter em-

ployed. The bars of Chicago blue and of the trypan dyes are slow to form and narrow, those of brom phenol blue appear quickly and are considerably broader, while with patent blue V barring is almost immediate, but so too is diffuse staining and the bars, very broad though they are, can be discerned for but a few minutes. We have ascertained the influence of the quantity of a highly diffusible dye on the gradient of distribution.

Windows were inserted at several places over the external oblique of 2000 gm. rabbits, and from  $1/2$  to  $1/10$ th the standard amount of patent blue V was thrown into circulation. The largest quantity mentioned gave rise as usual to diffuse staining with a superimposed barring; but the coloration took place slowly and the bars occupied not more than a third of the tissue. With  $1/4$ th the ordinary dose a still more tardy, general staining was produced and upon this, not a barring but a pronounced narrow tracery of color about the transverse venules. With  $1/7$ th the dose the muscle colored to the same slight extent everywhere, and the blood was soon rid of the dye. All these findings were confirmed at post mortem.

### *Significance of the Gradient*

The conclusion seems justified that the gradient responsible for barring with a highly diffusible dye brought in abundance to the tissue leads merely to its equal distribution when little is available. Inequalities develop only when so much dye is carried by the blood, or so indiffusible a dye, that not enough is lost along the capillary channel to counteract the influence of the gradient. Of our materials patent blue V most nearly approaches a normal food stuff in diffusibility. In water it spreads about as fast as dextrose but in gelatin less than one-third as rapidly. There is no reason to believe that a gradient which profoundly influences the distribution of highly various vital stains will fail of effect upon other substances. One may suppose that it acts to offset the progressive loss of normal stuffs along the capillary way, with result that the tissue is everywhere served to the same extent by the blood. But is any such supposition justified? Does there exist the need for an arrangement to equalize opportunity along the capillaries? The structure of the muscle vessels, when considered with the changes undergone by the blood coursing through them, provides an answer to these questions.

The groups of muscle capillaries which connect transverse arterioles with transverse venules vary in length from 0.43 mm. to 1.35 mm. in the adductor magnus, and average 0.69 mm. (1). In the external oblique they are as long. Krogh (11) ascertained that those which are open in resting guinea pig muscle have an average diameter of only  $3.5\mu$ . Corpuscles are deformed while passing through them. In injected and fixed rabbit muscle they range between 2.5 and  $5.5\mu$  (12). It follows that the capillary length is often several hundred times its breadth. The merest glance at an injected specimen poses the problem of tissue maintenance by such vessels (Fig. 7). Blood coursing through the hairlike channels, running the gauntlet of protoplasm that both takes and gives, is inevitably so different on emerging from what it was on entering that the segment of muscle fibre served from the distal portion of the capillaries would exist in a totally different milieu from that at their beginning were not conditions equalized in some way. The necessity for such equalization lies in the fact that each fibre can be only as strong as the weakest point in its length. One may urge that in working muscle the vessels are distended and tortuous, the current fast, there is a copious lymph flow, and contraction of the fibres, all aiding distribution. Nevertheless the blood when it reaches the veins is largely depleted of food materials, and is loaded with waste. In resting muscle many of the capillaries are closed off (11).

To compensate for the disadvantageous circulatory conditions some mechanism regulatory of exchange must exist along the capillaries, or else graded linear variations in the avidities and habits of the protoplasm of the muscle fibres, variations repeated with each successive relay of little vessels in the series that minister to its great length. The latter conception seems preposterous.

The possibility must be noticed that the stuffs in the blood may maintain the portion of fibre along the first portion of the capillary in a continual state of surfeit, so to speak, with result that this part has a negligible influence to deplete the blood and produce the need for a gradient. Such a condition of affairs occurring, not at the beginning but toward the end of the capillary in animals receiving patent blue V will explain why the barred coloration does not increase with the general deepening in hue, the early presence of dye in the barred region doubtless acting as a deterrent upon later escape there. But granting that under normal conditions distribution to the muscle along the first part of the capillary may perhaps be influenced in this way, there yet remains a further segment to be supplied from a blood that has undergone some depletion and will undergo more the further it travels along the tenuous vessel. The gradient disclosed by our experiments meets the needs of the situation.

*Significance of the Vascular Arrangement within the Liver Lobule*

The necessity for some arrangement to equalize opportunity where cells of a single sort are ranked along the capillary is clearly shown by the existence of a structural artifice for this purpose in the liver, as also by the untoward changes when the artifice proves inadequate, which frequently happens upon occasions of functional stress.

The cells of the hepatic parenchyma live in an equilibrium easily disturbed by alterations in blood flow, the result being a balanced hyperplasia and atrophy (13). By changes so produced the organ is normally molded from its shape in the embryo to that in the adult. Nevertheless under the ordinary circumstances of life the cells toward the center of the lobule, though served by blood that becomes progressively more venous as this center is approached, thrive in competition with their fellows near the source of supply. They are enabled to do so by the arrangement of the vessels. The little channels carrying the blood which serves the liver cords converge from the periphery of the lobule toward a central vein, and unite with one another, the consequence being that in proportion as this vein is approached more and more blood passes a cell in a given time. Thus, though increasingly impoverished of food stuffs and laden with waste, the blood can still tend the parenchyma adequately.

The distance from the periphery of the lobule to the center is not short. In the rabbit it averages 0.4 mm. in a straight line (14). When the animal is deprived of food an atrophy of the parenchymal cells takes place which is more severe the nearer the center of the lobule these cells are. It matters not that this atrophy has been traced to lessened function (15); for function like food is an opportunity provided by the blood in the form of materials. Not enough of these reach the central cells under the circumstances described for them to maintain themselves in competition with the peripheral elements. The value of the example over others innumerable, of peripheral, central and mid-zone lesions, which could be culled from the literature of pathology, lies in the simplicity of the conditions providing it.

The frequency with which opportunity is unequally distributed within the liver as evidenced by untoward parenchymal changes suggests that the vascular arrangement for distribution is unaided by a gradient of permeability along the capillaries. These let even protein through (16).

*Distribution from Capillaries of the Bladder*

Where capillaries run in an interlaced felt-work, near to one another and often in opposite directions so that the same cell is served

from different parts of several, there may be no need for a mechanism to equalize exchange. They are thus arranged in the outer layers of the urinary bladder.

The curve of the bladder is too great in the guinea pig, rat and mouse for satisfactory studies. But when the bladder of the rabbit is partially filled with warm milk, exposed under mica, and viewed by cooled, reflected light the more superficial vessels are clearly visible in an illuminated matrix, and one sees that the capillaries widen greatly as they approach the veins and that the blood stream slows concomitantly to such extent that first the flow itself, then the individual cells, become visible. The increased wall surface and the slowing stream would both tend to equalize opportunity along the capillary way. No gradient of vascular permeability has been demonstrable with our dyes, the tissue served by the network of vessels appearing to color everywhere at once. This one would expect under the conditions even in the presence of a gradient.

### *Relationship of the Vascular Structure to the Gradient*

The maintenance of a gradient of distribution in skeletal muscle despite circulatory changes entailing dilatation or great contraction of the vessels indicates that it has a structural basis; and the example of the liver suggests that this may be found merely in local differences in capillary number and size. We have made extensive studies to settle the matter.

Rabbits were etherized, the stomach, intestines and spleen removed through a median incision after ligation of the vessels, and the incision sewed shut. The sternum was split longitudinally, a cannula inserted in the ascending aorta, the right heart opened as a vent, and the animal washed blood-free with warm saline solution containing amyl nitrite. It was next immersed in saline solution at body temperature, a thin gelatin-India ink mass was injected under pressure, then a thick one, and finally enough gelatin-carmines to differentiate the arteries. At intervals the outlet for fluid (through the right ventricle) was obstructed, to aid distension of the capillaries, and finally it was tied off while the injection pressure was maintained. After transfer of the animal to ice-cold 95 per cent alcohol the abdomen was opened by severing the sutures. After fixation for 24 hours *in situ* the external oblique was dissected out and cleared.

In the thinner parts of the oblique of young rabbits the veins and arteries lie in practically a single plane (Figs. 2 and 3). Spalteholz portrays final arterial and venous trunks in regular alternation transverse to the fibres of the adductor magnus and coming off from parent vessels that run side by side. A similar final alternation is achieved in the external oblique but usually in more complicated fashion (Figs. 2 and 3).

Counts of the capillaries are best made in the long muscles. Spalteholz' drawings depict well the minute vascularization. Because of the differing levels upon which it is distributed photographs are unsatisfactory.

At an early period of alcohol fixation, before the injected tissue has become friable, one can tease out portions of individual fasciculi of the long muscles with vascularization intact. One sees after clearing them that relatively few capillaries are present in the region where the arterioles split up (Fig. 6). The latter divide into twigs, some of capillary magnitude, some slightly larger, and the larger ones fork again, often repeatedly, and now into capillaries. The number of these vessels reaches its maximum midway between transverse arteriole and venule, and thence until the venule is neared it does not alter significantly (Fig. 6), the average increase being less than one in fifty, as shown by some forty comparative counts. Cross connections are few. Some of the capillaries enter the venous trunk directly while others may come together just before it is reached, uniting into radicles parallel with the fibres. Even in injected specimens such radicles are almost always shorter and broader than the corresponding arterial twigs, a fact Spalteholz noted. In his precise and comprehensive account no mention is made of any increase in the capillaries as the venule is neared.

None of the several authors measuring injected capillaries mentions any progressive widening along them (12), though a recognition of such widening would have been essential to interpretation of their findings. In our own specimens the capillaries appeared of remarkably even bore.

The vascular arrangement provides a reason for the limitation of the colored bars to the distal half of the region between arterial and venous trunks. The channels from which dye can conceivably pass into the proximal half of the tissue are relatively few, and other things being equal the staining here should be slighter and slower than further on. The barring occurred where the capillaries were relatively numerous and constituted the sole source of supply for the tissues. The graded increase in color as the venules were approached cannot be explained by any increase in the number of capillaries nor, apparently, in the total expanse of vessel wall. But injected and fixed specimens yield only approximate data. In Krogh's report on the capillaries of living guinea pig muscle no mention occurs of any widening toward the venules, and none in v. Hösslin's (12) study of fresh muscle tissue. When in our own work the blood was darkened with India ink, or with dyes that had as yet not passed into the tissue, the muscle should have shown some trace of barring if the capillaries had enlarged toward the venous end, and they should there have been most readily discernible.



Instead it appeared diffusely overcast and under the microscope the capillaries were like threads of even calibre. Poorly diffusible dyes escape only from the distal portion of the capillaries during no inconsiderable time after their injection (Fig. 8), and highly diffusible ones pass out most abundantly there, precisely where the blood is poorest in dye and its pressure lowest. An increase in wall surface vast enough to account for such happenings could not be overlooked. In the absence of it one must ascribe the greater escape of dyes as the capillary end is approached to a graded lessening of the barrier between blood and tissue, that is to say, to an increasing permeability of the capillary wall.

### *The Literature of Distribution along the Capillaries*

The problem presented by the length of the capillaries in its influence upon exchange with the tissues has attracted singularly little attention. One might say that these vessels have been viewed in cross section, seldom in three dimensions.

Schade (17) has invoked the influence of the capillary pressure, that of the blood colloids, and various other factors, in an elaboration of Starling's surmise that the passage of substances into the tissues takes place predominantly through the first portion of the capillary wall with resorption as the major activity further on. Krogh has criticized (18) the hypothesis destructively, but Landis (19) has procured evidence which might be cited in its support. He frequently observed a filtration of dye-stained fluid from the first portion of the mesenteric capillaries of frogs. The vessels had been exposed for more than an hour under Ringer's solution before the observations were begun. In the frog much lymph is continually produced, by filtration through the capillary walls generally. In resting mammalian muscle the amount formed is almost nil (20).

The dyes of our experiments appeared most abundantly in the region where conditions would, at first thought, seem least favorable on Schade's hypothesis. Nevertheless, it is possible to explain certain of the phenomena in terms of his view, if this be taken to imply an active extravascular flow in the direction of the venule. It might be assumed, for example, that the escape of poorly diffusible dyes circulating in great quantity actually takes place from the first part of the capillary but so slowly that no color is visible anywhere until the escaped dye, passing along the outside of the vessel to its further end, fails for some reason to pass into the blood as abundantly as it had emerged, with result that it accumulates in a colored band. But how explain on this basis the fact that the least quantity of poorly diffusible dye that will cause perceptible staining yields characteristic bands of the usual

dimensions at the end of the capillary? Can one suppose that there exists a selective impediment to return just where according to Schade everything should be highly favorable to it?

Highly diffusible dyes stain the tissue next the transverse collecting venules only slightly less than that at the distal end of the capillaries. Is one to suppose that the pigment responsible for this staining escapes primarily in the proximal capillary region and is carried by extravascular ways with the swiftness of the blood itself to reach its eventual situation outside the veins? If the staining is deemed to have come about by direct extension through the vein wall, can one refuse to admit the evidence for an even greater extension from the adjoining portion of the capillaries?

These and the many other respects in which our findings fail to conform with Schade's hypothesis are less impressive as objections to it than is the length and shape of the capillary itself. To assume that the escape of substances takes place preponderantly from the first portion of a hair-like vessel  $\frac{1}{2}$  to  $1\frac{1}{2}$  mm. long and resorption from its further part is to go afield for a concept which would interpose between the tissue and the blood serving it an obstacle to the equalization of cell opportunity even more considerable than that which would exist were the narrow channel everywhere permeable to the same extent.

### *The Zone of Effective Vascular Permeability*

Physiologists are accustomed to think of the vessels as of a three-part system, arteries to bring, capillaries to exchange, and veins to collect once again. In the main this classification undoubtedly holds. Yet our experiments with patent blue V prove that the wall of the arterioles of muscle will let through a coloring matter somewhat less diffusible than dextrose, while the venule walls are readily penetrated by it. What are the limits of permeability along the vascular system? Obviously they must vary with the substance under consideration, being wide for materials of great penetrative ability, such as  $\text{CO}_2$  and urea, and contracted to the vanishing point (at the venous end of the capillary) with substances of very large molecule. Their extent for this or that substance need not be discussed; but it is important to determine the collective outcome of the penetration of the vessel wall by normal stuffs, to learn in other words the limits of effective permeability along the vascular way.

The paucity of vessels in the walls of the larger arteries and their absence from small ones led long ago to the inference that the walls must be nourished partially, or wholly, by direct exchange with the blood. Several authors (21) have described a rapid penetration of highly colloidal dyes into the arterial wall. Veins

are said to stain even more quickly. When a segment of the aorta or renal artery is separated from its surroundings and coated with wax it survives and abnormalities develop only next the wax (22). An effective exchange between the blood and the tissue of the wall evidently takes place; but the high blood pressure and the possible presence of "stomata" may render the instance special.

Where there are *vasa vasorum* in the adventitia the effective influence of the blood in the main lumen cannot extend as far as this layer. Whether it reaches beyond in the case of the arteries and veins with avascular walls has not been directly determined. The small venules of human skin are walled by a single layer of cells, and capillaries are so infrequent that the venules may be supposed to serve in their stead, as Krogh points out (18). Lewis (23) believes that the capillaries, the venules that are simple endothelial tubes, and the "weakly equipped arterioles" all act to nourish the cutaneous tissue; and Kreyberg (24) has stated a like view for the skin of the mouse. But the inference that venules and arterioles must function as capillaries because they are walled only by endothelium is not entirely warranted. Vessel walls cannot be considered to have the same permeability because they are merely one cell thick, as the present work sufficiently attests.

The regularity of the minute vascularization in skeletal muscle enables one to perceive gaps in the capillary spacing. Hence it should be possible to tell whether any of the venules and arterioles serve the fibres effectively, since where they fail to do so capillaries must be present to perform the task.

On teasing out the injected and partially fixed muscles of the rabbit (*gracilis*, *adductor magnus*) one sees that the largest vascular trunks running transverse to the muscle fibres lie between the fasciculi and give off branches to these on one or both sides, which branches plunge transversely amid the fibres and ramify. Those to superimposed fasciculi do not quite coincide in position and therefore the colored bars seen in a thick layer of fresh, translucent muscle are broader than is the actual distribution of the stain along the individual capillaries (Fig. 11). Intrafascicular arterial twigs that run parallel with the capillaries are far more frequent than venules (Fig. 6).

In paraffin cross sections the injected capillaries stand forth as dots arranged at the angles of the roughly polygonal muscle fibres, while larger black discs represent the venules and arterioles (Figs. 4 and 5). A light staining with hematoxylin and picric acid greatly aids identification of the structures. One can readily perceive that those vessels larger than capillaries which lie like these latter, within the ultimate muscle fasciculi and next the fibres, are spaced at about the same distance from the nearest capillaries as if they actually were such. Where they exist no other source of nourishment for the tissue can be found. All have walls only one cell thick, but the cell nuclei are nearer together in some of the vessels, doubtless the arterioles. Along the margins of the fasciculi capillaries are relatively

infrequent as would follow from the circumstance that here the demands on the blood are diminished by nearly half. But even here a difference is to be noted between regions adjacent to a small vein or arteriole and those further off. The latter regularly exhibit many more capillaries.

Specimens teased or cut in the direction of the muscle fibres give corroboratory information. The individual fibres pass directly through the forkings of the transverse arterial trunks, and while some few capillaries pass with them, the majority do not, but join the arborization. Where the fibres traverse this latter they must of necessity depend in great part on the blood of its small branches. The venous twigs are more close set than the arterial, and more capillaries pass through the arborization, indicating that it plays a smaller part in maintenance of the tissue.

Interfascicular arterioles equipped with a layer of muscle are not infrequently accompanied by a capillary; but even around these and the venules of corresponding magnitude such vessels are infrequent. But here the determinations are no longer aided by an almost diagrammatic vascularization.

Specimens from cats and guinea pigs yield the same findings.

The conclusion seems justified that in muscle the last arterioles serve the tissue about them at least as well as do the capillaries. Where they course these vessels are dispensed with. The venules share to a minor extent in the task of muscle maintenance.

### *Contours of the Gradient*

Our dye experiments have disclosed a mounting gradient of vascular permeability in muscle, which first becomes effective along the finest arterioles and trends almost vertically upward with the transition to the capillaries. Along these hair vessels the gradient, far from flattening out, mounts again steeply to reach its peak where they join to form the least venules, declining along the veins rather gradually.

The most casual calculation of the area of wall through which exchange can take place from the blood of arterioles, venules and capillaries, respectively, discloses how negligible this is save in the case of the vessels last mentioned, and that of the smallest collecting venules. When the gradient of vascular permeability is considered in the light of this fact one perceives that the arterioles and venules can have but a slight share in serving muscle. The capillaries after all carry out the major task of its maintenance. Yet the function is not so sharply localized to them as general belief would have it. Someone has happily said that in the animal body form fits function like a glove; but when there is more than one function and these not strictly super-

imposed, the glove must bulge a trifle here and pinch there. Thus it is with the blood vessels of muscle, which have the widely disparate tasks of carrying fluid and cells over long distances under pressure and of facilitating exchange with the tissue at one region only along the way. There is no matter for surprise in the realization that through the walls of the finer vascular branches some incidental, beneficial, leakage takes place.

A vast deal has been written on capillary permeability, with the assumption implicit in the term that permeability is the same all along the little vessels. This is certainly not true of those supplying voluntary muscle. Limitations of space forbid discussion of facts in the literature which support the concept that at not a few situations the permeability of the capillary wall increases progressively as the venules are approached and is greatest at the junction with them. In succeeding papers this concept will be expanded.

#### SUMMARY

The permeability of the capillaries in the skeletal muscles of mammals increases progressively along their course and is greatest where they pass into the least venules. The gradient of permeability is so largely independent of functional states as to give grounds for the view that it is determined by inherent local differences. Through the gradient opportunity is equalized along the capillary. In the liver lobule this object is accomplished by an artifice of arrangement whereby the blood flow past the cells is increased with their distance from the source of supply. In the urinary bladder the interlacing of capillaries, their progressive widening, and a consequent gradual slowing of the blood flow act to achieve the same end. Here a gradient of permeability has not been demonstrable.

Where cells of different sorts are served by a slender capillary, their differing requirements may render unnecessary any provision to equalize their opportunities; but where shortcomings in local maintenance will reduce the efficiency of an entire fabric, as the muscle fibre, and where cells of like character live competitively along the same channel, as in the liver, some arrangement must exist to ensure an even distribution of the services rendered by the blood. In situations of the kind last mentioned the immediate environment of the individual cell,

the "milieu interne" of Bernard, is not only kept as constant as possible but it must be the same, by and large, for all of the cells.

The task of serving voluntary muscle is not strictly limited to the capillaries. The intrafascicular arterioles and venules act so effectively to sustain the tissue about them that where they run no capillaries are supplied.

It is a pleasure to thank the laboratory staffs of du Pont de Nemours and Co., the General Dyestuff Corporation, Hynson, Westcott and Dunning, the National Aniline and Chemical Co., the Sandoz Chemical Works, and the Winthrop Chemical Co. for a most generous provision of dyes and data.

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## EXPLANATION OF PLATES

## PLATE 14

FIG. 1. Edge of the gracilis of a 700 gm. rabbit, photographed by transmitted light after clearing. The vessels had been injected with an India ink-gelatin mass to show the alternation of transverse arterioles and venules. The striae between are the injected capillaries.  $\times 12$ .

FIG. 2. Thinner portion of the external oblique of a 700 gm. rabbit, prepared in the same way. The entire depth of the muscle is shown. The injected capillaries are represented by groups of parallel striations. The muscle bundles are somewhat separated.  $\times 12$ .

FIG. 3. Another region of the external oblique, to show arterial and venous trunks that course with the muscle fibres, not across them, to approach each other and spread suddenly into a skein of capillaries.  $\times 12$ .

FIG. 4. Cross section of the semitendinosus of an 800 gm. rabbit injected with ink-gelatin mass. The vessels appear as black dots. The larger of those which represent the intrafascicular arterioles and venules are in general spaced at the same distance from the nearest capillary as if they functioned as such. The capillary next one of them is a branch from it.  $\times 140$ .

FIG. 5. A similar specimen to show the same fact, from one of the lumbar muscles of a rabbit.  $\times 200$ .

FIG. 6. Drawing of a final vascular unit in a small group of fibres of the adductor magnus of a rabbit, showing the typical arrangement in unusually pronounced form. The vessels had been injected with ink-gelatin mass and the muscle teased out and cleared. The fibres are not shown.  $a$  = arteriole,  $v$  = venule. The capillary number reaches its maximum about midway between them. The long, arteriolar branchings are characteristic, as also the stumpy venule in which the capillaries terminate rather abruptly. One of the capillaries in the neighborhood of the arteriole has been broken and bent back.  $\times 70$ .

FIG. 7. For comparison with Fig. 6. Final vascular unit in the vastus lateralis of a rabbit injected with ink-gelatin mass.  $a$  = arteriole,  $v$  = venule. Those capillaries only are visible which lie in a single plane.  $\times 130$ .

## PLATE 15

FIG. 8. Fern-like color pattern in the external oblique of a 2700 gm. rabbit killed by cutting the carotids 30 minutes after injection of the standard amount of Chicago blue 6B. The greater part of the muscle is wholly unstained. Photographed between glass plates by a combination of transmitted and reflected light. Natural size. The black spot is an artefact.

FIG. 9. Situation of the dye in such a preparation as shown by the microscope. Each of the venules transverse to the muscle fibres lies in the midst of a cloud of color. The tissue about the arterioles (indicated by arrows) which alternate with the venules is wholly unstained.  $\times 11$ .

FIG. 10. Similar findings in the external oblique of a rabbit injected with the standard amount of Chicago blue 6B and killed with ether 3½ hours later. The localization of the staining to the distal capillary regions is well shown. The arterioles lie in relatively unstained tissue. The veins contain much dark blood and the transverse venules had been purposely distended with it by pressure. One is entered from a single direction by capillaries and venous radicles, and on this side only has staining taken place. Near the base of the transverse venous trunks where no capillaries enter no staining has occurred.  $\times 17$ .

FIG. 11. Gracilis of a rabbit killed by cutting the carotids 19 minutes after injection of the standard amount of Chicago blue 6B. The muscle was photographed *in situ* by reflected light, with a glass plate over part of it. Natural size.

FIG. 12. External oblique of a cat brought into shock by Cannon's method, injected with the standard amount of brom phenol blue, and killed 3 minutes later. During the injection the low blood pressure rose transiently. The dye is seen to be less narrowly localized than Chicago blue, but confined like it to the region supplied from the further portion of the capillaries. Photographed between glass plates by a combination of transmitted and reflected light. Natural size.

### *Schemata to Illustrate the Differing Distribution in Muscle of Dyes of Differing Diffusibility*

#### PLATE 16

#### *Schema I*

A. A parallel artery and vein are shown from which trunks arise alternately that run transverse to the muscle fibres and are connected by capillaries (not shown).

B. *Distribution of a Highly Diffusible Dye (Patent Blue V) in a Bled Animal.*

(a) The slow blood stream has as yet carried the dye only to the beginning of the capillaries where it has at once begun to pass out into the tissues. (The tufting is exaggerated, occupying too large an area.)

(b) A bar of dye forms with the distributing arteriole as its axis.

(c) The dye has now progressed to the end of the capillaries, coloring the tissue along them evenly, thus obliterating the bars.

(d) Bars of deeper hue are seen superimposed upon the general coloration, owing to an especially great escape of dye along the further capillary region. Some of the stain has passed into the venous blood. The zone of color along the main collecting vein shows that its wall has been penetrated by the dye, despite the small quantity of it present in the venous blood as compared with the arterial.

(e) All color differences are lost in an intense general staining.



## PLATE 17

*Schema II**C. Distribution of a Poorly Diffusible Dye (Trypan Red).*

(a) The dye circulates through all the vessels but is as yet escaping only about the ends of the capillaries. (The tufting is exaggerated, occupying too large an area.)

(b) The dye passing out along the distal portion of the capillaries generally has produced a narrow, colored bar with the collecting venule in its midst.

(c) Some staining has taken place along the entire length of the capillaries, yet that in the distal region is still especially pronounced.

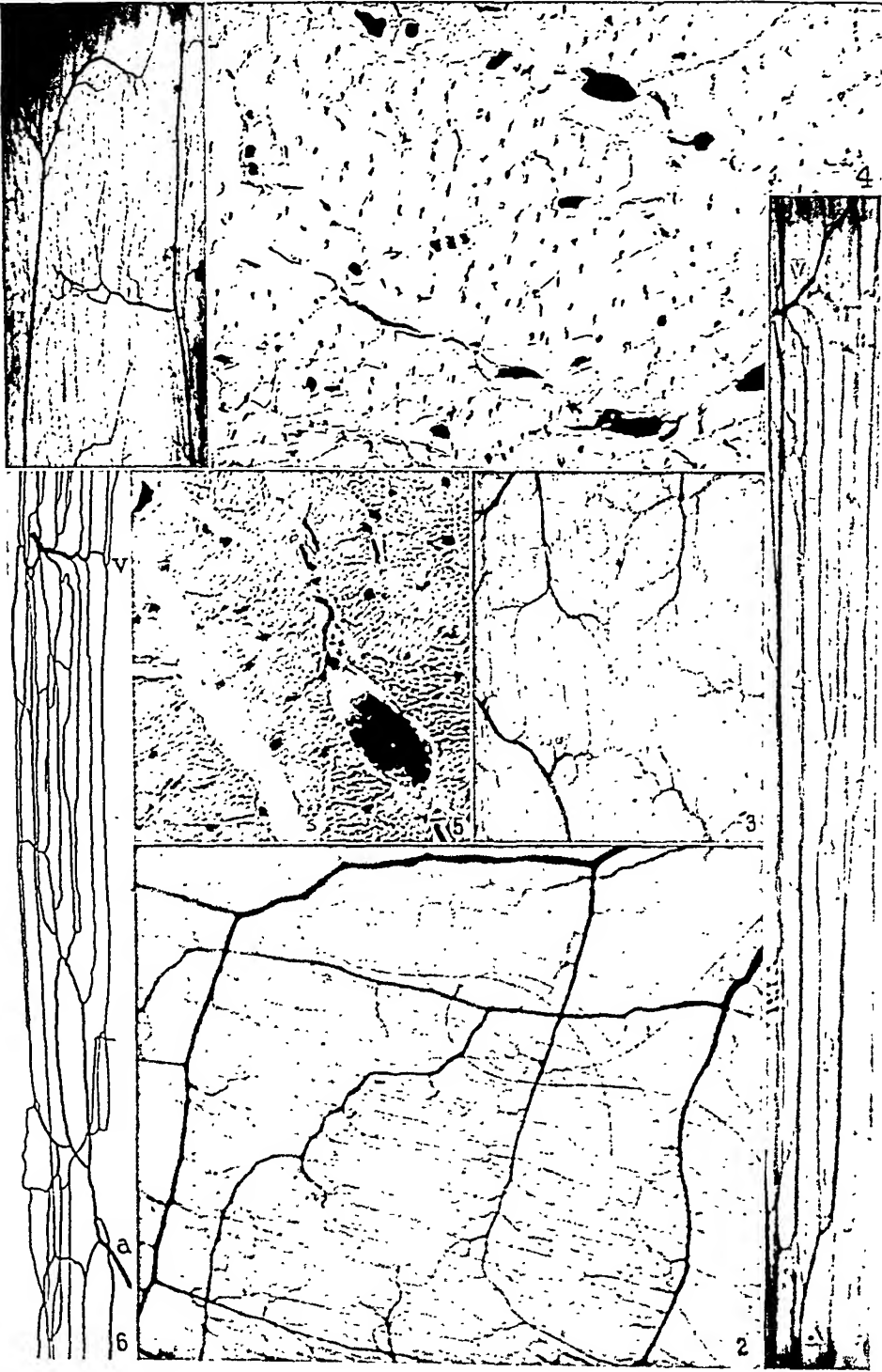
(d) The progressive escape of dye all along the capillaries has obliterated the local differences.

*D. Distribution of a Moderately Diffusible Dye (Brom Phenol Blue).*

(a) The dye has escaped from the distal portion of the capillaries forming a bar, although the tissue elsewhere is unstained as yet. In consequence of the local escape of dye almost none has reached the venous blood.

(b) The dye has escaped along the entire length of the capillaries, but the staining in the distal region is still especially pronounced, and a zone of color immediately next the relatively large collecting vein shows that it too is permeable to the dye. The venous blood is still poor in dye as compared with the arterial.

*Final stage* (not depicted). All color differences are lost in an intense general staining.

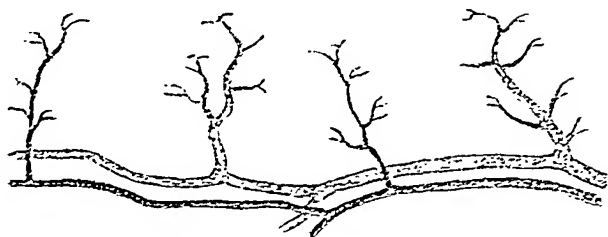


(Rous *et al.*: Gradient of vascular permeability)

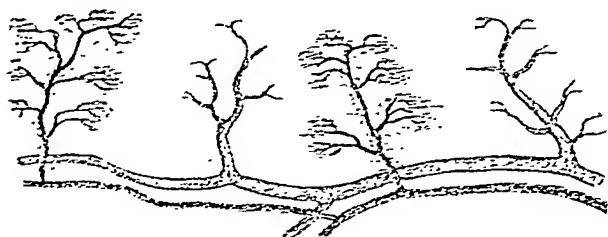


(Rous *et al.*: Gradient of vascular permeability)

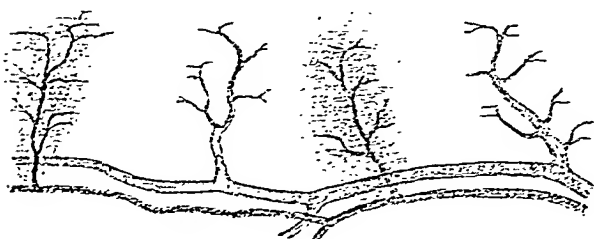




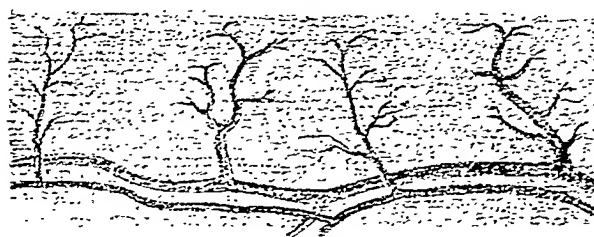
A



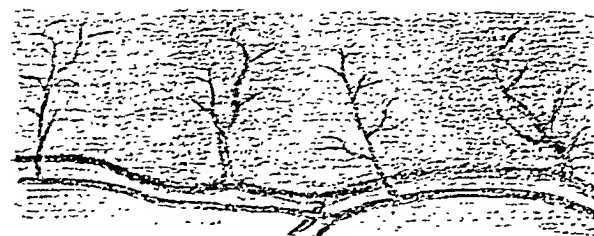
B<sub>(a)</sub>



B<sub>(b)</sub>



B<sub>(c)</sub>

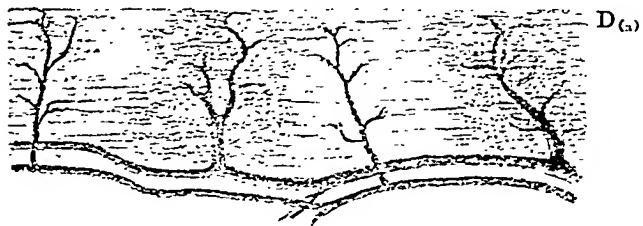
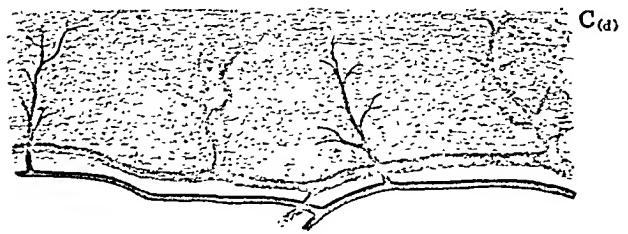
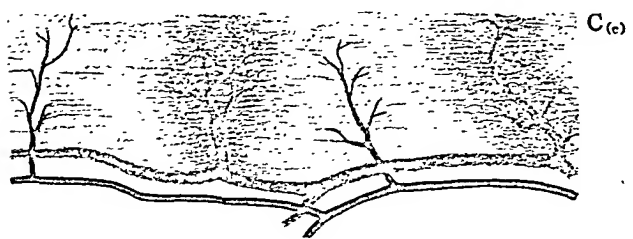
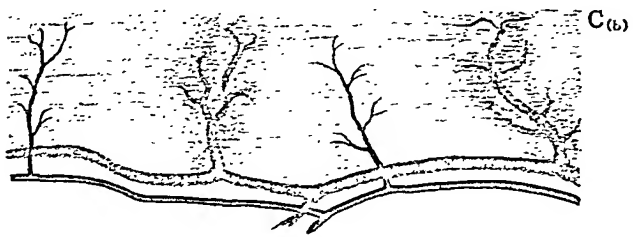
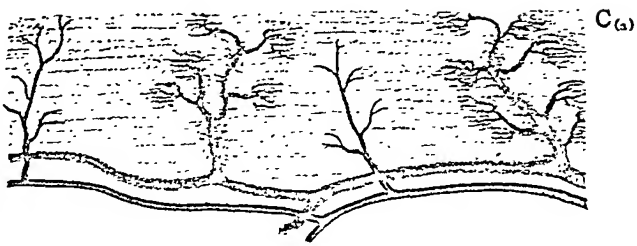


B<sub>(d)</sub>



B<sub>(e)</sub>





Schema II

(Rous et al's Gradient of vascular permeability)





# BIOLOGICAL STUDIES OF THE TUBERCLE BACILLUS

## I. INSTABILITY OF THE ORGANISM—MICROBIC DISSOCIATION

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PLATES 18 TO 26

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It has become increasingly evident during recent years, that bacteria along with other living forms are subject to modification, embraced under the term of "mutation." The recognition of types of "mutation" is now general among systematic bacteriologists, as is the employment of such terms as "smooth" and "rough" to define differences in colony formation appearing in the "mutating" bacterial species. The literature on this subject has been assembled in papers and monographs and is now readily accessible so that it will not be reviewed in this paper. We refer those seeking the literature to the article by Hadley (1), but it is desirable to direct attention especially to the pioneer work in this field of Lehmann and Neumann (2), Neisser (3), Massini (4), Baerthlein (5), Arkwright (6) and DeKruif (7).

Just as the observations on bacteria in general have shown bacteria to undergo "mutation," so have the studies on tubercle bacilli, in tissue and cultures, shown them at times to be pleomorphic in structure. It is not uncommon to see in preparations "coccoid" granules, rods, beaded and branching rods. Reviews of the literature on this subject may be found in the articles on the tubercle bacillus by Kolle, Schlossberger and Pfannenstiel (8), Kahn (9) and others.

### *Tubercle Bacillus Cultures*

Our purpose in this paper is to deal with the gross cultural characteristics of the tubercle bacillus, with special reference to the topography of colony formation and the virulence according to colony type. No mention will be made of the pleomorphism which may or may not play a part in the dissociation phenomena.

When a pathogenic acid-fast organism is cultivated on favorable media, generally the growth appears white at first. As time goes on, it becomes a cream color at the center of the crowded area and dark orange color as it becomes older. The growth may be wrinkled and

heaped in masses; or show large folds having a smooth surface; or it may be spreading and smooth. Not all cultures develop with the same rapidity; some develop profusely in a few weeks, while for others a much longer time passes before growth becomes visible.

Variations in virulence have been noted by many workers. For the last twenty years we have used the well known Saranac Laboratory human tubercle bacillus culture  $H_{37}$  for studies in infection and immunity. Up to a few years ago the virulence of this organism was very constant and apparently a small number of organisms when inoculated into guinea pigs, was sufficient to produce progressive disease. Within the last three or four years a diminution of virulence has been observed such that in some cases we failed completely to infect animals even with a heavy suspension. From time to time we have noticed a variation in antigenic properties of this organism, with special reference to the complement-fixation reaction. Some antigens were anti-complementary and low in fixing properties, while antigens prepared from a different lot of  $H_{37}$  cultures were very potent.

When the tubercle bacillus  $H_{37}$  is cultivated on liquid media, *i.e.*, veal, synthetic or potato broth, two types of growth may be seen; veil-like filament and small wrinkled heaps. The veil-like growth is confluent, spreads in very thin layers and in time covers the surface of the medium, gradually pushing its way up the side-walls of the flask. The growth coheres and it is very difficult to remove a small portion without disturbing the whole mass. Chromogenicity is generally absent. The second growth appears in small isolated islands connected by a veil-like growth. After some time it becomes very wrinkled and dry. At first it is white, and as it ages, creamy, and finally it assumes an orange color. The production of pigment is much greater in the center of the mass than at the periphery. The growth is fragile and brittle and any portion can be lifted without disturbing the adjoining one. Cultures of the first sort can be triturated to an evenly diffused suspension with no difficulty, while those of the second sort are much more difficult to put in a fine suspension.

If we start with a colony having distinct cultural characteristics, there will probably be a number of variants from the original mother colony upon subsequent cultivation. Two, perhaps three, or more variants may develop from the original colony and these may differ

from each other not only in cultural structure, but also in tinctorial characteristics, virulence and other characteristics.

In the following pages we shall describe in detail the methods used in dissociating various acid-fast organisms. Observations are recorded just as we have seen the various colonies. No attempt will be made to interpret the various stages of their development. For the earlier observations the reader is referred to previous studies by the authors (10).

### *Culture Media for Dissociating the Tubercle Bacillus*

*Gentian-violet-glycerol-egg medium* with a slight modification was used throughout this investigation. The gentian-violet dye exerts some bacteriostatic action on the tubercle bacillus, a loss which is more than compensated for by the usefulness of the dye. The dye has two functions: (a) its dark color makes a satisfactory background, on which white colonies stand out prominently for observation and photography, and (b) it to some extent prevents external contamination. Even with all precautions contamination, especially by the molds, cannot be entirely prevented.

*The Modified Medium.*—*Meat juice:* 500 gm. of chopped lean veal or beef are infused in 500 cc. of 15 per cent solution of glycerine in water in a refrigerator. After 24 hours the meat is squeezed through cheese cloth and the juice is collected in a sterile beaker. *Eggs:* The shells are washed well and dried, and left in 70 per cent alcohol for  $\frac{1}{2}$  hour. They are removed from the alcohol and placed on a sterile towel to dry. They are then broken, emptied into a sterile beaker and mixed well with a sterile glass rod. One part of the meat juice is added to two parts of the eggs by volume. *Gentian-violet:* A 1 per cent alcoholic gentian-violet solution is added to make a final dilution 1 to 30,000 (1 cc. of dye to 300 cc. of the medium). The mixture is well stirred and filtered through sterile gauze to remove coarser particles. 35 cc. of the medium are put in Petri dishes (100 x 15 mm.), and inspissated the first day at 85°C. until completely coagulated, removed from the inspissator and put in the incubator for a gradual cooling (face down). On the second day the dishes are again put in the inspissator at 85°C. for a second inspissation for 1½ hours, after which they are again put in the incubator at 37.5°C. for 3 days to test their sterility. While still in the incubator, rubber bands are slipped around the edge of the plates and they are then removed to room temperature. This step in the preparation of the medium is very important for the following reason: At 37.5°C. the air in the Petri dishes expands and in part escapes with result that when the dish is removed to room temperature room air enters it carrying contaminating bacteria. This difficulty can be eliminated by the use of rubber bands. All plates must be incubated face down to prevent water condensation on the upper lid of the Petri dishes. We cannot emphasize too strongly the importance of this step.

*Preparation of the Rubber Bands (See Plate 18A).—*Automobile inner tubes (30 x 3½ inches), preferably new, are cut into bands 1½ inches wide. These can be shaped into proper form by using wood discs 15 cm. in diameter and 1.5 cm. thick. The bands are stretched over the disc so that one-third will cover the sides of the disc and the remainder will cover the bottom. The discs must have a central hole of  $\frac{3}{8}$  inch. Twenty-five to thirty such discs over which the rubber bands have been stretched can be threaded on a rod through the hole, a nut put on each end and tightened, autoclaved for 1 hour at 15 pounds pressure, and cooled. The bands when removed from the discs are found to have shaped themselves so that they fit satisfactorily over the Petri dishes.

*Preparation of the Suspension for Plating (See Plate 18B).—*A small portion of a recent 12 to 14 day growth, preferably from fluid medium, is removed aseptically with a sterile platinum wire spade and triturated with the same on the inside wall of a sterile Wassermann test tube containing 3 to 4 cc. of sterile saline of pH 7.8. The trituration is done just above the fluid level. From time to time the platinum wire is dipped in the saline to carry more moisture for trituration. After complete trituration the tube is inclined and the triturated material washed into the saline. The suspension is filtered twice through two layers of sterile Whatman No. 5 filter paper. The filtrate is then diluted five to ten times for the human tubercle bacillus, while the bovine and avian require ten to twenty times. The filtrate is inoculated on the surface of five or ten plates made as described above, usually four to six drops from a capillary pipette being used for inoculation. The rubber bands are fitted around the edges of the Petri dishes. Adhesive tape may be used for labels. Rubber bands prevent desiccation of the medium as well as contamination. When a plate is taken out of the incubator, the rubber band should never be removed until it has completely cooled.

*Funnels for filtration (see Plate 18B)* may be made from discarded test tubes, 18 to 20 mm. in diameter. The upper portion of the funnel can be made 1½ to 2 inches long, pulled out in the flame to about 3 to 4 mm. Two layers of Whatman paper No. 5 are inserted in the funnels, suitably folded to make a cup form. For consecutive studies a number of these funnels containing the filter paper can be prepared, placed in a jar, sterilized and used as wanted.

Growth may not appear until the third week. The best time to study the colonies is after 6 to 8 weeks when they have partially matured. For the study of the finer structures of the colonies we have used a Bausch and Lomb low power binocular microscope with drum type objectives. After a plate has been removed from the incubator for study the lid of the plate may become foggy and interfere with vision. This can be remedied by warming the lid of the plate for a few seconds over a Bunsen burner.

### *Dissociation of the Bovine Tubercle Bacillus B<sub>1</sub>*

The bovine culture which we have successfully dissociated is known as B<sub>1</sub>. It was isolated by Dr. E. R. Baldwin in 1904. The organism has been cultivated on

glycerine potato, glycerine egg and veal broth. The virulence has been constant for rabbits and guinea pigs until a few years ago since when it has occasionally failed to produce progressive disease in rabbits.

A suspension of 2 weeks' growth was prepared by the method described above and inoculated on the surface of gentian-violet-egg plates. After 4 weeks, isolated colonies appeared on the plates. One of the colonies (a) was perfectly round, raised, opaque, with a smooth surface resembling a moth-ball. It was easily removed from the surface. The second colony (b) was large, spreading, raised at the center, dry and wrinkled. The (a) colony was readily emulsified, while the (b) colony was suspended with difficulty (10).

The separate types of isolated colonies were emulsified, the suspensions filtered and new plates prepared. After due time, colony (a) instead of being like a moth-ball, was flat, spreading and appeared very much like a star. In the middle of each colony there was a small nipple-like growth with a smooth surface, which was surrounded by a darker pigment forming a circle and from this central zone the growth radiated in the form of large folds toward the periphery in a rosette form. The periphery of the colony was very irregular and raised. The colony (b) had a different topography, the central zone being raised with numerous irregular folds and at places appearing like a honeycomb. The periphery was flat and veil-like with perfectly smooth edges spreading over the medium. The colony (a) of the second subculture was difficult to emulsify, while (b) could be prepared in suspension very readily. The different physical properties relating to emulsification probably are due to the lipin content or they may have a different isoelectric point.

The two principal colonies which we have just described were difficult to separate at times and their characteristics were sometimes very confusing. Very often colonies appeared on the plates differing from the two colonies already described. It must be kept in mind that there may be some intermediate colonies which develop from time to time and play some part in the life cycle of the organism.

From the preliminary observations reported here we were convinced that dissociation of the tubercle bacillus does take place and that our methods up to this time were inadequate to make a further study until we could find a medium which would stabilize the development of the organism and retard the dissociation. In other words it was required for further studies that the colonies should be of uniform topography and readily separated.

Recently we have modified the medium with the object of obtaining uniform colony structure. The base of the medium was gentian-violet-egg to which sodium glycocholate and sodium taurocholate were added. Sodium taurocholate in the proportion of 0.25 per cent was found to be most suitable.

When the two colonies dissociated from the bovine tubercle bacillus

were cultivated on this medium, they presented characteristics which could be differentiated without any difficulty. One of the colonies was perfectly round, moist, like a moth-ball, opaque, with smooth surface, easily emulsified and on subsequent subcultures on plates developing a round and flat colony. The other colony was large, flat opaque, changing with age to cream yellow, moist and with smooth surface. It emulsified with difficulty and on subsequent subculturing developed flat colonies (see Plate 19, 1, 2, 34).

The former we shall now call "S" and the latter "R." Organisms of the "S" colony when inoculated into guinea pigs, regardless of the route used, produced generalized tuberculosis in a short time, the disease spreading rapidly throughout the viscera. A few drops of a suspension of 5,000,000 organisms of this colony when introduced into the conjunctiva, without injury, produced conjunctivitis and vitreous opacity with photophobia within 4 weeks. The cervical lymph nodes could be palpated and in many instances the inguinal lymph nodes could be palpated after a short time. The animals reacted to tuberculin within 12 to 15 days and the intensity of the reaction was approximately 15 x 15 mm. with a central necrotic area. Animals inoculated intracutaneously, intraperitoneally, intracardially and intratesticularly developed generalized tuberculosis and death in 3 to 6 weeks. This colony also produced rapid, progressive tuberculosis in rabbits after inoculation with a small number of organisms. The disease was very extensive and tubercle bacilli could be found in the bone-marrow 2 months after the inoculation.

When a suspension of the "R" colony was used in a strength similar to that of the "S" colony, it was found that the disease did not progress but remained localized. When a few drops of a suspension of 5,000,000 bacilli of "R" colony were instilled into the conjunctiva of a normal guinea pig, the result was wholly unlike that observed with the "S" colony. The animal failed to react to tuberculin up to the fourth week and no lesions were observed in the eye. In some animals which were killed for comparison, the only ones found were healed tubercles in the posterior auricular lymph nodes. Intratesticular, intracardiac and intracutaneous inoculations produced lesions which in the majority of instances healed. Lesions were observed in rabbits inoculated by various methods, but they were not like those observed with the "S"

colony. No tubercles were found in the lungs, spleen or liver, up to 8 weeks.

The foregoing observations on guinea pigs and rabbits which were inoculated with the two colonies, were of brief duration (4 months). The results might have been different if we had allowed the animals inoculated with the "R" colony to live longer. We consider this of great importance and believe that some of our animals inoculated with the "R" colony after 12 to 18 months or more might have died of tuberculosis because of a reversion of the bacilli in the animal to the "S" type. This will be referred to in a later paper.

### *Dissociation of the Avian Tubercle Bacillus A<sub>1</sub>*

The culture of avian tubercle bacillus studied was A<sub>1</sub>, an organism which has been in our possession for many years. It is slightly pathogenic for chickens and only occasionally produces progressive disease in rabbits.

A suspension from a 2 weeks' growth of a plain egg culture was prepared by the usual method and filtered. The filtrate was picked up with a sterile capillary pipette and six drops seeded on the surface of gentian-violet-egg plates. The ordinary gentian-violet-egg medium may be used satisfactorily in dissociating the avian tubercle bacillus, sodium taurocholate not being necessary for it. After several weeks, a number of single colonies appeared on the plate, the majority of which were flat, slightly opaque, raised in the center, and the periphery appeared translucent. Upon careful examination with a low power lens, a small number of somewhat darker, opaque, round colonies could be seen scattered over the surface.

This second colony is like a moth-bali, moist, glistening and easily emulsified in an even suspension, growing profusely in alkaline synthetic medium but without any diffusion of pigment into the fluid. The first colony is flat, spreading and very similar to the "R" colony of the bovine culture. It grows more profusely in synthetic medium with an acid reaction and produces a slight amount of pigment (see Plate 20, 3, 4, 5 and 6).

The two colonies on gentian-violet-egg medium revealed some very striking changes when kept in the incubator for a period of 8 months. The "S" colony first developed into a moth-ball and after several months spread and assumed a flat appearance, remaining flat for another 3 months. After 7 months from the time of seeding from the mother colonies secondary daughter colonies appeared in the form of papillae. They have, at times, a worm-like appearance sprouting in all directions, the growth being sprinkled with a few moth-ball papillae. The periphery of the mother colony is moist, perfectly smooth and cream-like in color. Subcul-



tures from the periphery developed mostly colonies with typical "R" characteristics, while the worm-like growth or the moth-ball papillae developed some "S" colonies (see Plate 20, 1 and 2).

The "S" organism is highly pathogenic for chickens,  $\frac{1}{3}$  mg. inoculated intravenously producing septicemia and death in 30 days. The "R" colony is not very virulent, and will not produce the same type of disease.

### *Dissociation of the Human Tubercle Bacillus*

An attempt was made to dissociate a human type of tubercle bacillus known as H<sub>37</sub>. In the preceding pages we have mentioned that this organism was formerly highly pathogenic for guinea pigs, but that in the last 3 or 4 years in some instances it has failed to produce progressive disease. Such fluctuations of virulence appear to be connected with the media used.

It was noted that the diminution of virulence occurred when the organism was cultivated on veal broth with an acid reaction. On the other hand, if cultivated on Proskauer and Beck synthetic medium which is slightly buffered and has an alkaline reaction, no loss of virulence was observed. Ten bacilli from such a culture very often produced tuberculosis in guinea pigs, leading to death in from 100 to 150 days. It was very difficult to dissociate H<sub>37</sub> by the method used in dissociating the other strains. The difficulty, in all probability, could be explained by the facts (1) that we used a suspension prepared from Proskauer and Beck medium cultures, in which virulent "S" organisms predominate, and (2) that the colonies which appeared on the gentian-violet-egg plates from a filtered suspension, differed decidedly from those seen in bovine, BCG or avian cultures. Each variant developed colonies of different topography, and there was no fixed characteristic which could help us to separate the colonies. They were unstable, and the gentian-violet-taurocholate medium was of no value. Another method had to be devised. Remembering that this organism lost its virulence when cultivated on glycerine-veal broth of acid reaction, we decided to cultivate the organism on Sauton fluid medium buffered at pH 6.6. After a number of cultural passages through this medium, followed by cultivation on gentian-violet-egg plates, we have dissociated two or perhaps three colonies which can be seen in Plate 21, 1, 32, 2, 3, 26 and 4. The supposed "R" colony is very waxy, much raised, with large folds. The periphery is sharply outlined, and emulsifies with difficulty in salt solution. It produces a chromogenic soluble substance in acid synthetic media. When cultivated on solid media, it at first appears creamy-yellow, a characteristic noted of "R" colonies of other bacteria, but as it ages it becomes yellow. The "S" colony is flat, spreading, composed of small wrinkles, appearing at times like ground glass. This colony is

easily emulsified in salt solution of pH 7.2 and grows best on synthetic media of pH 7.6.

The above is the description of the two main colonies. Recently, we have observed other variants which will be described in a subsequent study.

### *Dissociation of Bacillus Calmette-Guerin (BCG)*

The result of dissociation of BCG was published in a recent article (10). At that time the two extreme "R" and "S" colonies were illustrated and fully described.

It has been noted by the workers engaged in the study of this organism that cultures of BCG distributed by the Pasteur Institute several years ago were more virulent for guinea pigs than the recent ones. In a previous study (10) we offered an explanation for the loss of virulence after prolonged cultivation on glycerine-potato-bile medium. We pointed out that only the "R" strain develops readily on that medium and that "S" does not grow well and no visible colonies can be seen in cultures. Therefore, by the process of gradual elimination, the undissociated cultures at present harbor only a very small number of "S" organisms.

Culture BCG 359 (which Petroff obtained in the fall of 1928 from the Pasteur Institute) was more difficult to dissociate than the three cultures which had previously been obtained. Out of some thirty-five plates made from the same suspension, only one plate showed two distinct and one questionable "S" colonies. For this reason we advocate the use of a large number of plates (30 to 50) in attempting to dissociate BCG.

The four following methods have proved successful in our hands in the dissociation of BCG:

1. The original BCG culture was cultivated on Proskauer and Beck's synthetic medium with a reaction of pH 7.4 to 7.6 and subcultures were made every 2 weeks. After six to eight subcultures many "S" colonies could be isolated by the plating method.

2. Rabbits were inoculated intravenously or intraperitoneally four times at intervals of 3 to 4 days with a 5 mg. suspension of undissociated BCG killed in the water bath at 100°C. for  $\frac{1}{2}$  hour. 4 weeks from the beginning of the inoculation the rabbits were bled aseptically and the clear serum was added to Proskauer and

Beck's medium in the proportion of 10 per cent. After a preliminary incubation to test sterility, the surface of the flasks was seeded with the original BCG culture and subinoculation made on similar medium every 2 weeks. If a transplant is made from an eight to ten passage on gentian-violet plates, the "S" colony can be separated. At the same time the whole culture acquires virulence for guinea pigs.

3. The virulence of the original culture has been increased, with the "S" colony predominating, by cultivation on synthetic medium containing 10 per cent normal rabbit serum. However, the transformation is much slower in our experience.

4. The "S" colony can be dissociated from abscesses formed in guinea pigs. Guinea pigs were inoculated with 10 mg. of the original BCG culture. 6 to 8 weeks later the same amount was again inoculated subcutaneously. Several weeks after the second inoculation, plate culture prepared from the abscesses revealed a number of "S" colonies.

Dissociation has been accomplished successfully in our hands by any of the four preceding methods. Workers following them will find that single colonies will appear on the plates after from 40 to 60 days. On careful examination, several different colonies with distinct cultural characteristics will be found present in the plates.

We shall describe at first the two widely different colonies which we have studied more extensively than any others.

The "R" colony (see Plate 22, 39) appears waxy, with slightly raised center, sloping gradually towards the periphery. Sometimes the colonies appear in rosette form, but the general characteristic is that of a coil of small intestines. The periphery is clear cut, raised and does not extend into the medium. This colony is at first slightly chromogenic, becomes with age a dark orange, and is very difficult to emulsify in salt solution of pH 7.4. On Sauton's synthetic medium it grows very profusely in small islands. A mass of growth can be lifted very easily without disturbing the surrounding colonies. The organism grows well at pH 6.5 to 6.8. It will grow for the first and second subculturing on alkaline broth for the probable reason that a small amount of the medium is transferred from the original culture; but after the third subculturing the colonies cease to develop.

This colony grows very readily on Calmette glycerol-potato-bile medium. It appears first in small round pebble-like colonies, the surfaces of which are perfectly smooth. They gradually increase in size, occasionally reaching 1 to 2 mm. in diameter. The surface of this colony remains perfectly smooth for the first month. 4 to 6 weeks later a small granular papilla appears at the end, gradually covering the whole surface with secondary growth. At the end of the third month several shoots of coarser papillae develop. Eventually the original colony is covered with this secondary growth, entirely changing its appearance.

The "S" colony is composed of minute irregular wrinkles and the whole structure is much more delicate than the "R" colony. It appears very much like honey-

comb. The periphery is veil-like and extends into the medium, will not grow on glycerol-potato-bile, grows best on Proskauer and Beck medium pH 7.8, is easily emulsified and is more sensitive to gentian-violet-egg medium (see Plate 22, 40).

The pathogenicity of these two colonies has been described in our recent paper (10) and to avoid repetition we have omitted the experimental data on animals.

### *Reversibility of "R" to "S" Virulent Organism*

Recently, we (10) have described in detail the method used in changing the "R" into "S" colonies. Since that time we have repeated the experiments sufficiently often to say here that when the "R" colony is cultivated on media containing anti-"R" serum after the 8th to 10th cultural passages, the offspring of the original colony becomes "S."

### SUMMARY

The recent advances in the study of the other bacteria with application to the dissociation phenomenon, have been applied in the study of acid-fast organisms. For some time, we have realized that the term "dissociation" as employed at present, is not adequate to explain the instability and subsequent variation which occur in cultures. But for uniformity of bacteriological nomenclature, we have adopted the term until a better one is coined. In describing the "R" and "S" colonies, we have had to depart somewhat from the general usage of these terms, that is the "R" meaning rough, and "S" smooth. The colonies of acid-fast organisms are relatively varied and complex. It seems better to employ the letter "R" to indicate greater resistance to environment and relative avirulence; and "S" to indicate colonies which are more sensitive to environment while possessing for certain species relatively great virulence. The terms "rough" and "smooth" apply directly only to avian tubercle bacillus, when cultivated on plain gentian-violet-egg medium. The avirulent colony isolated from this culture is flat and somewhat rough in appearance. The virulent is perfectly smooth, round and resembling a moth-ball. The physical properties are different. They have been fully described elsewhere.

When the bovine "R" and "S" are cultivated on plain gentian-violet-egg medium, differentiation is very difficult. At times they are

almost indistinguishable, but the addition of 0.25 per cent sodium taurocholate to the medium, alters completely the topography of the colony. The "S" appears in perfectly round smooth moth-balls, and the "R" in larger, spreading and somewhat rough colonies.

Lacking suitable media, the human tubercle bacillus H<sub>37</sub> has been more difficult to dissociate. After 2 years' study, using various media, we have been able to dissociate two types of colonies; but as the animal experiments are not yet completed, very little more than that can be said at present.

We have dissociated two extreme types of colonies from four BCG cultures obtained from various sources. Each of these four cultures has revealed the same types of colonies. For details the reader is referred to a recent paper (10). In this publication we have included photographs taken from time to time in order to keep a record of our observations. When studying the photographs, the reader will notice considerable variation in some of the colonies. Unquestionably, there are more than two types of colonies developing during the life cycle of the organism, but at present we have considered and confined ourselves to only the two extreme types, one which can produce progressive disease, leading to the death of the animal, and the other which is but slightly virulent, and sometimes not at all so for susceptible animals.

Full details of the technique employed by us have been described in the test. Anyone attempting to duplicate the work must strictly adhere to the technique described. *Departures from it may lead to failure.*

The underlying factors favoring dissociation are not yet clearly understood. We believe that every single bacillus contains the two components, "R" and "S." If the environment is favorable for the development of the "R" component, the offspring will be "R's," although the original organism may be "S." Conversely, if the environment is favorable for the "S" and not for the "R" component the "S" will develop. For example, if an avirulent "R" colony obtained from the avian bacilli is cultivated on egg medium, which is favorable for the organism, the offspring after a suitable length of time will develop "S" colonies.

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## EXPLANATION OF PLATES

## PLATE 18

A, process used in preparation of the rubber bands. a, properly cut rubber band  $1\frac{1}{4}$  inches wide. c, front view of rubber band stretched over the wooden disc. d, same, back view. e, number of such discs with stretched bands are strung on iron rod, tightened and autoclaved for 1 hour at  $121.3^{\circ}\text{C}$ . b, properly shaped rubber band.

B, process used in the preparation of suspension of organism for seeding. a, funnels. b, the organism is triturated just above the liquid level. c, Petri dish with the rubber band properly applied.

For details, the reader is referred to the text.

## PLATE 19

Colonies of bovine tubercle bacilli B<sub>1</sub>. 1, 2, 34 and 38 are on 0.25 per cent taurocholate gentian-violet-egg medium plates, 37 and 3, without taurocholate,  $\frac{1}{4}$  normal size.

1, the moth balls are "S," the flat are "R" colonies, age 150 days. 38, "S" colony, age 298 days, secondary variants have developed. 34, typical "R" colony, age 68 days. 2, typical "S" colony, age 68 days. 37 and 3, are "R" and "S" colonies cultivated on medium from which the taurocholate has been omitted; differentiation is very difficult.

## PLATE 20

Colonies of avian tubercle bacilli A<sub>1</sub>, on gentian-violet-egg medium plates; 1, 3, 4,  $\frac{1}{4}$  normal size; 2, 5, 6, magnification 7  $\times$ .

1, "S" colony, secondary growth and papillae, age 286 days. 2, same as (1), magnified 7  $\times$ . 3, typical "S" colony, a subculture from the papillae, 1-a, age 40

days. 4, typical "R" colony, a subculture from the periphery of 1-b, age 40 days. 5, higher magnification of (3). 6, higher magnification of (4).

#### PLATE 21

Colonies of human tubercle bacilli H<sub>37</sub> on gentian-violet-egg medium plates,  $\frac{7}{8}$  normal size.

1, undissociated mostly "S," few typical "R" colony structure has developed after 3 months, age 168 days. 32, ? "R" colony with autolyzed center, age 86 days. 2 and 26, "R" colonies, age, former 168, latter 100 days. 3, "S" colony which developed "R" in the center periphery is "S," age 86 days. 4, typical "S" colony, age 86 days.

#### PLATE 22

Colonies of BCG on gentian-violet-egg medium plates,  $\frac{7}{8}$  normal size.

39, Strain 3 "R" colony, age 67 days. 40, Strain 3 "S" colony, age 86 days. 1, Strain "Park," secondary veil-like growth (a) can be seen shooting under the mother colony. Subcultures from such growth developed "S" like structure, age 242 days. 4, Strain 3 undissociated. The structure is similar to (1), the secondary growth can be seen in (b), age 210 days. 3, Strain "Park," probably intermediate colony, age 242 days. 14, Strain 3 undissociated. This is very similar to (3), age 210 days.

The original cultures, dissociated and illustrated in Plates 22, 23, 24, and 25 were obtained from the following sources. No. 3, Pasteur Institute strain obtained in the spring of 1927. No. 359, Pasteur Institute strain, given in person to one of the authors in the fall of 1928. No. "Park" strain, a culture sent to Dr. Baldwin by Dr. W. H. Park, New York City.

#### PLATE 23

Colonies of BCG on gentian-violet-egg medium plates,  $\frac{7}{8}$  normal size.

20, subculture from undissociated Strain 3 organisms which has been cultivated through 7 passages on synthetic medium containing 10 per cent normal sera. Majority of the colonies are "S." Few of them have developed in the center an "R" structure, age 283 days. 21, Strain 359, at the beginning these were typical "S," as they aged "R" structure developed in the center, periphery continues to be "S," age 215 days. 22, this is unexplainable at present, some parts are "S" and the others look like "R," age 110 days. 9, Strain 359, undissociated, secondary growth can be seen developing from the original colonies, age 240 days. 16, Strain 3 undissociated, consisting of "R," "S" and intermediate, age 240 days. 5, Strain 359 undissociated, the growth is moist with autolyzed zone, age 110 days.

#### PLATE 24

Colonies of BCG on gentian-violet-egg medium plates,  $\frac{7}{8}$  normal size.

12, Strain "Park" S?, colony, age 130 days. 2, Strain 359, undissociated secondary veil-like growth, a and b. 18, Strain 3 undissociated; this plate was

seeded from a synthetic anti-sera medium culture. Majority of colonies on this plate are "S," age 210 days. 6, Strain 359. This colony erroneously has been described by Kraus, Tzekhnovitzer and Zeyland as "S" colony. Although it is smooth in structure, it is not the true "S" colony which we have described in the text, age 110 days. 17, Strain 3 "S" colony which has been cultivated for a long period on acid medium. The organisms after such treatment have mutated and changed into "R," age 110 days. 13, Strain "Park" "S" colony, age 110 days.

#### PLATE 25

Colonies of BCG on gentian-violet-egg medium plates,  $\frac{2}{3}$  normal size.

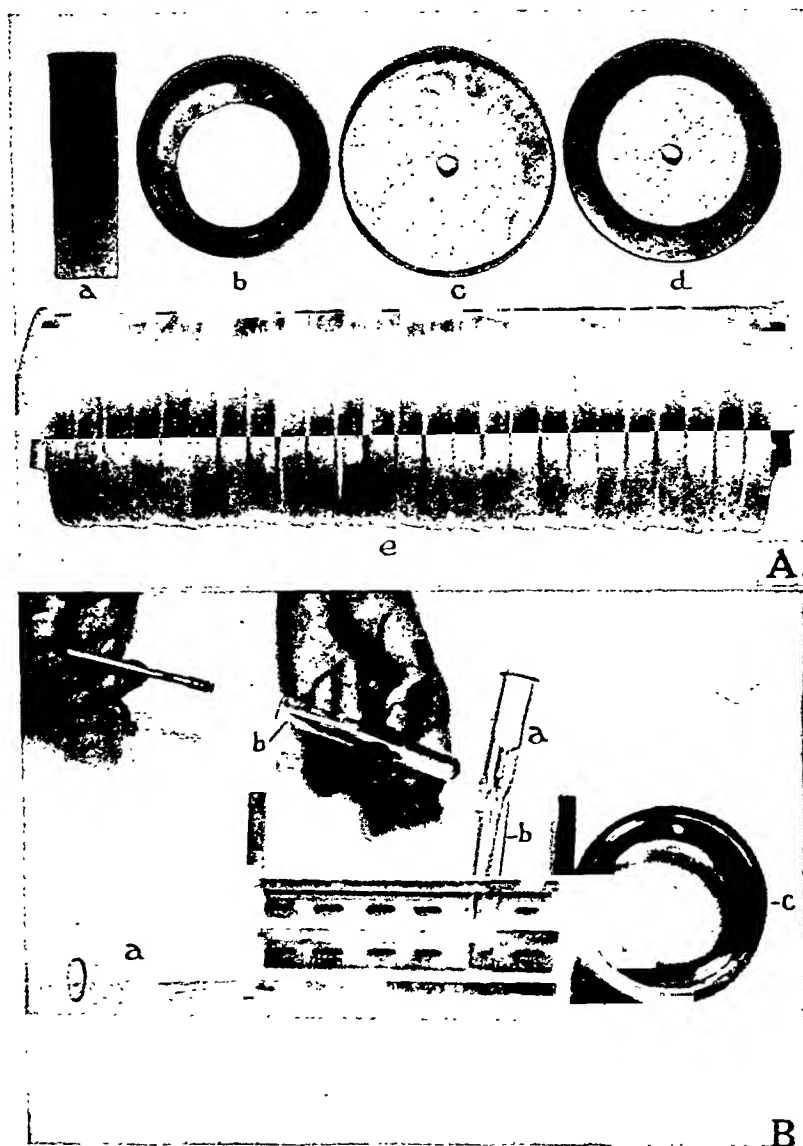
10, Strain 3 "S" colony which first developed "S" structure and as aged developed "R" structure in the center, age 281 days. 19, Strain 3 undissociated. It is a subculture from anti-sera growth; periphery "S" and the center is "R" structure, age 281 days. 11, Strain 3 "S" colony. Three zones can be seen, the center is "R," periphery is "S," the intermediate zone is undetermined at present, age 154 days. 7, Strain 3 undissociated organisms, giving same characteristics as (11), age 156 days. 8, Strain 359, colonies on sodium taurocholate medium, age 150 days. 4, Strain "Park." Note the secondary veil-like growth (a) shooting under the mother colony. Such growth upon subculturing develops many "S" colonies, age 210 days.

#### PLATE 26

Cultures of tubercle bacilli isolated from six patients, directly on gentian-violet-egg medium plates, using sodium hydrate digestion. Every one of these cultures revealed two types of colonies. Magnification 7 $\times$ .

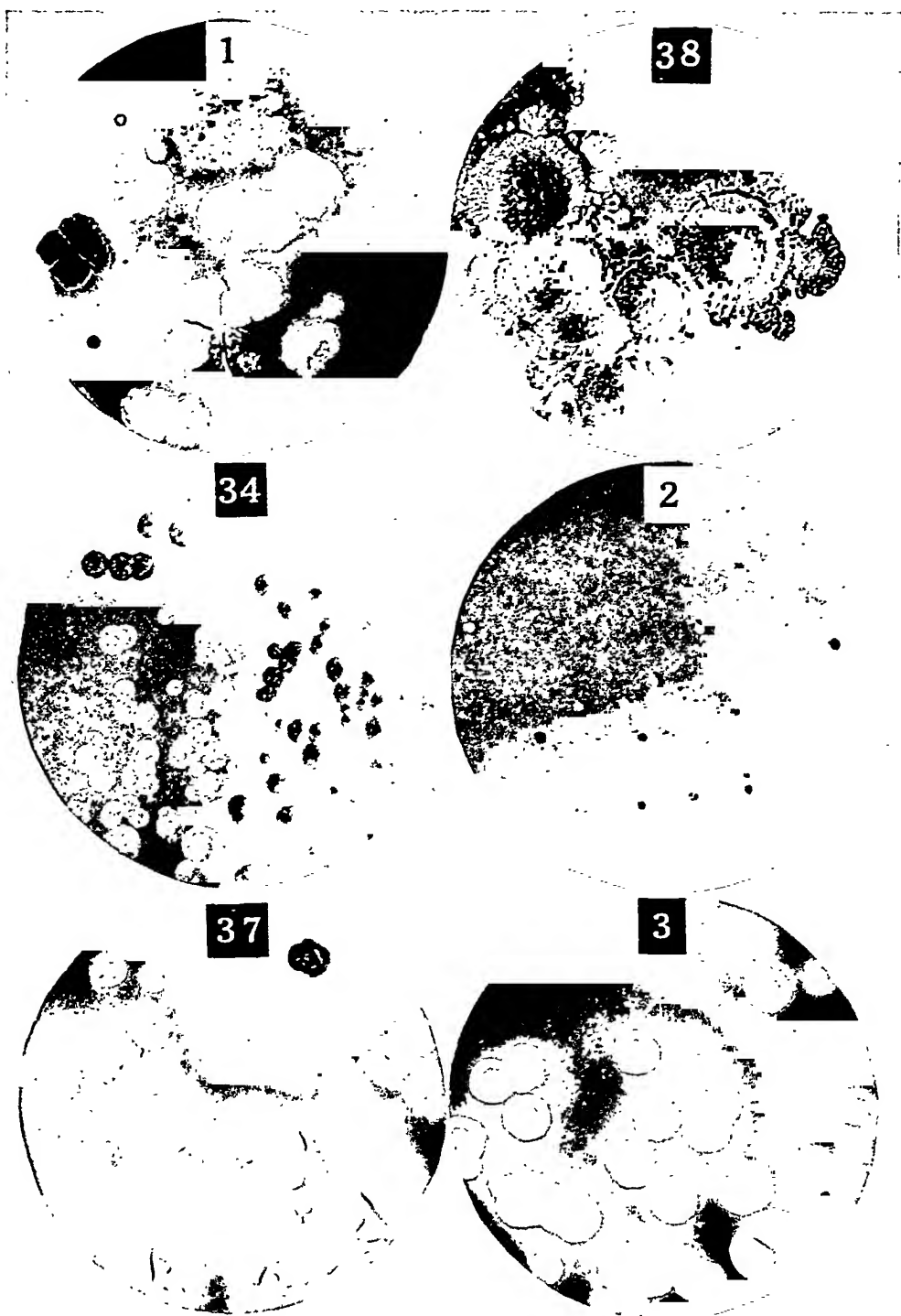




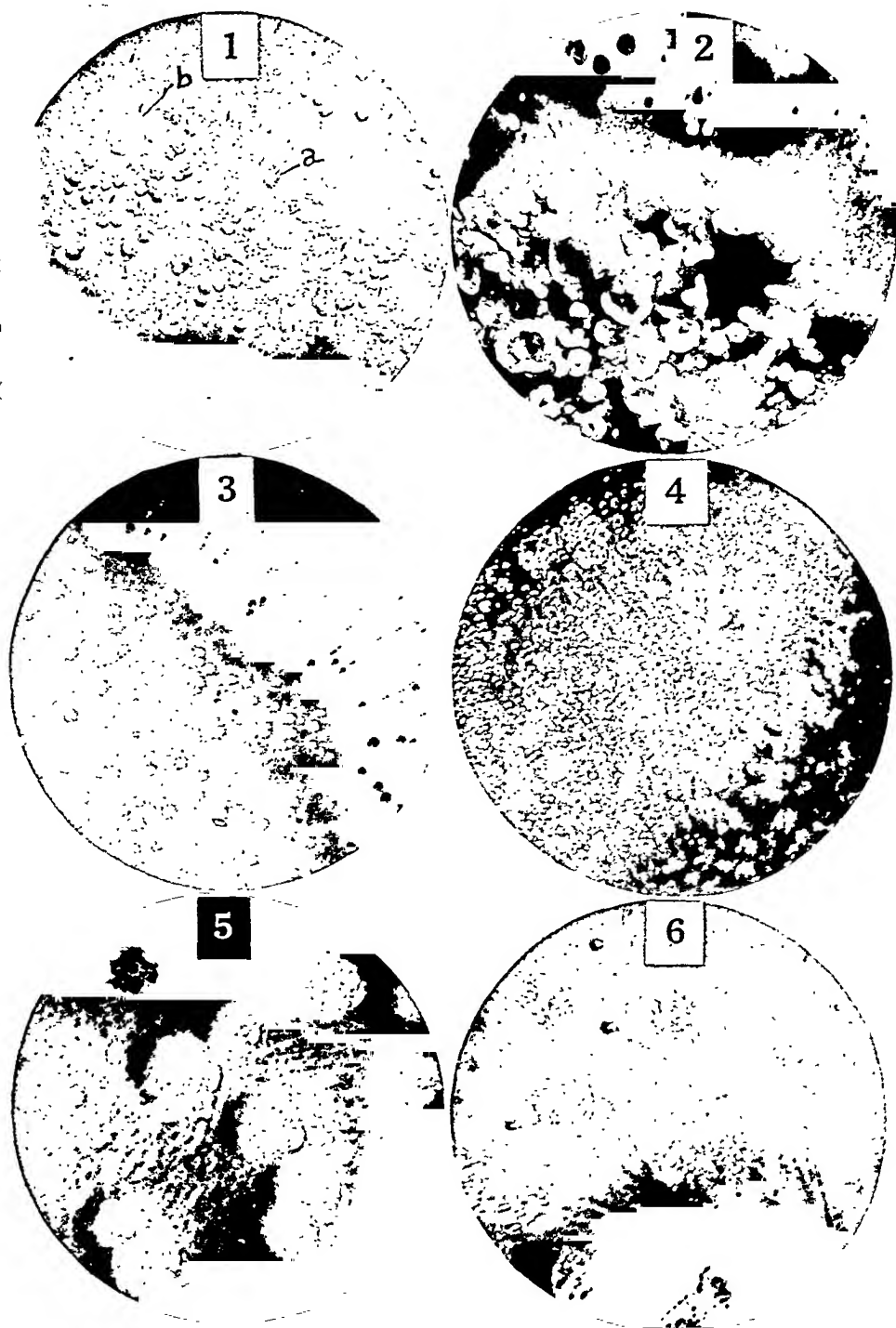


(Petroff and Steenken: Tubercle bacillus. I)

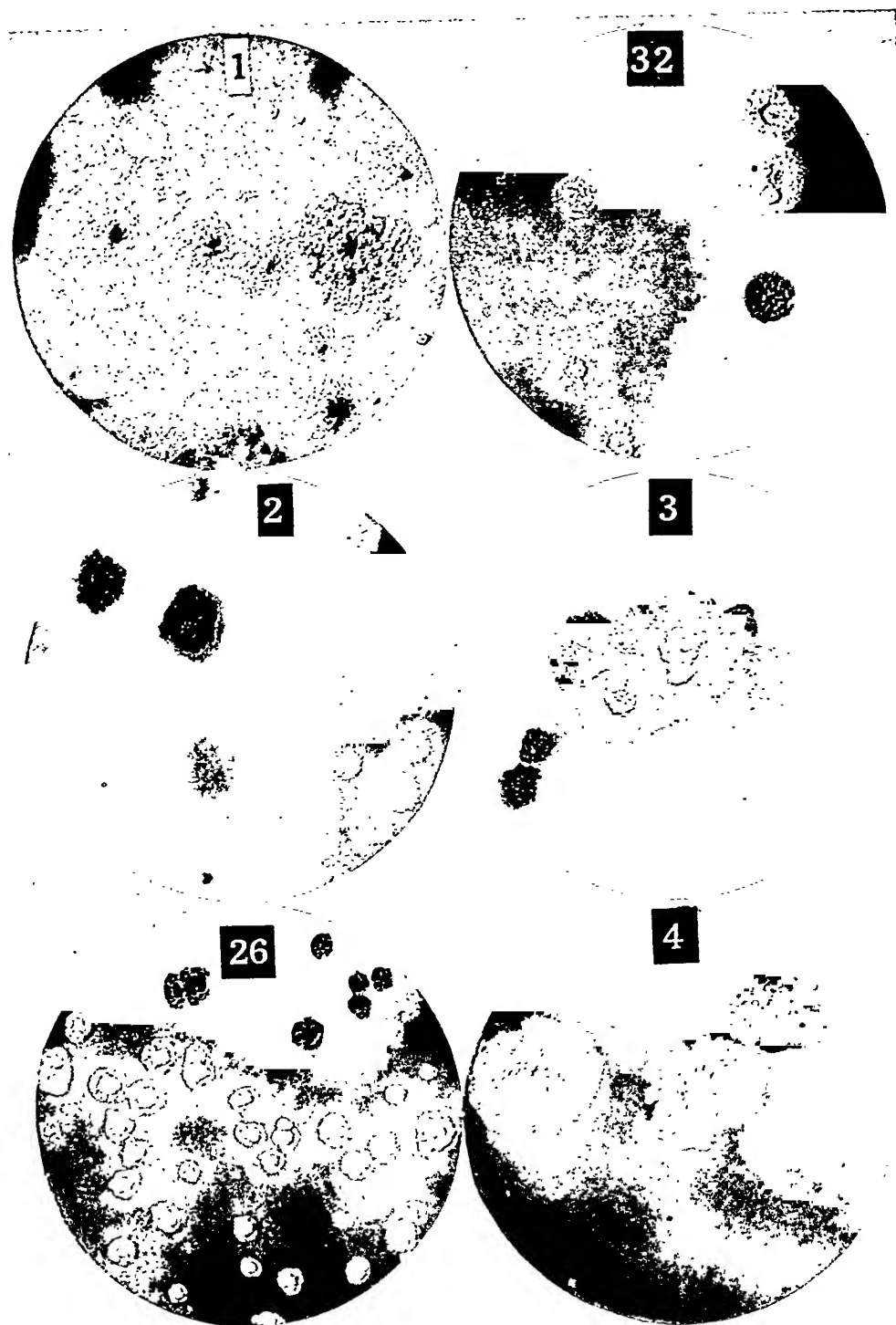






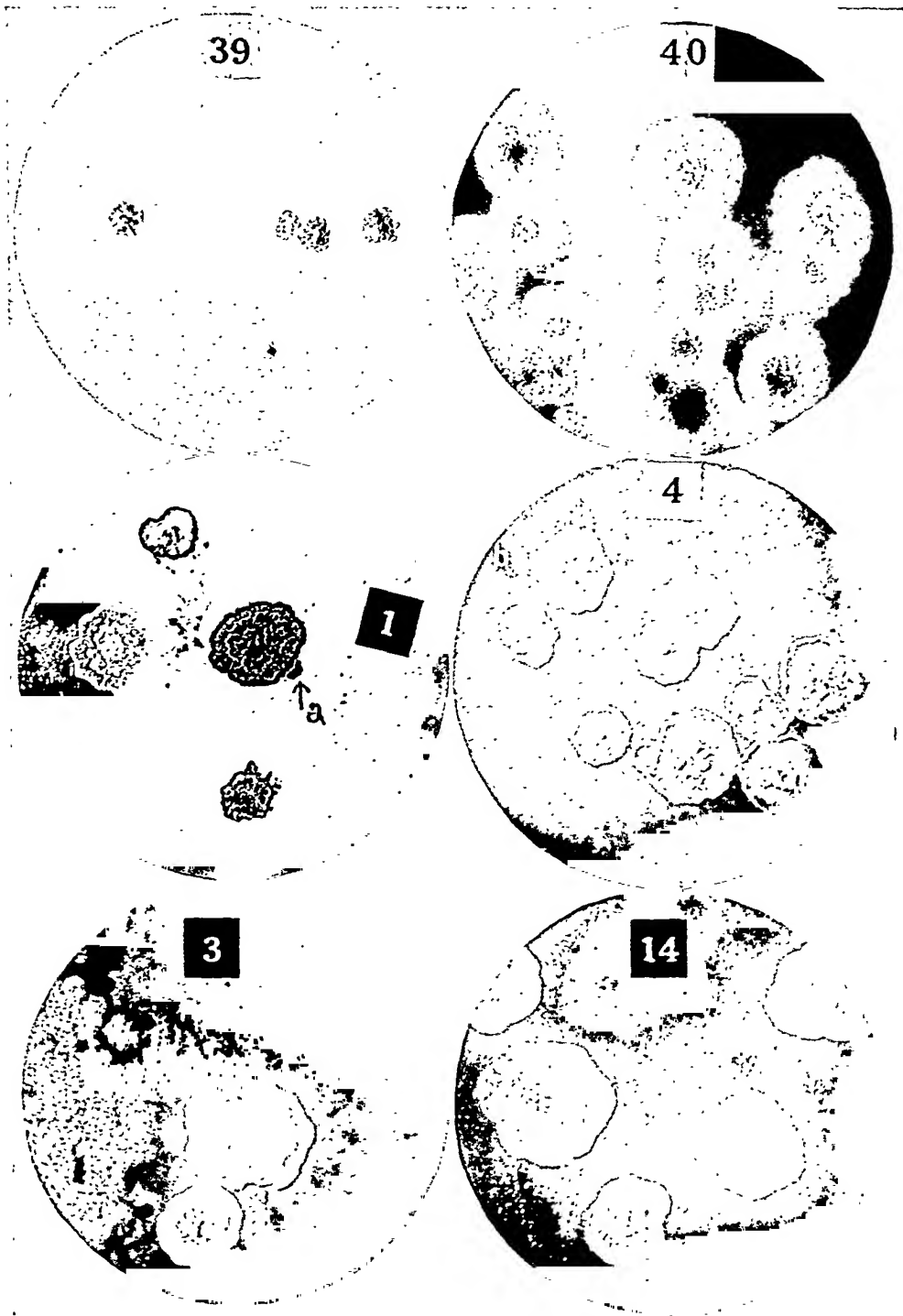






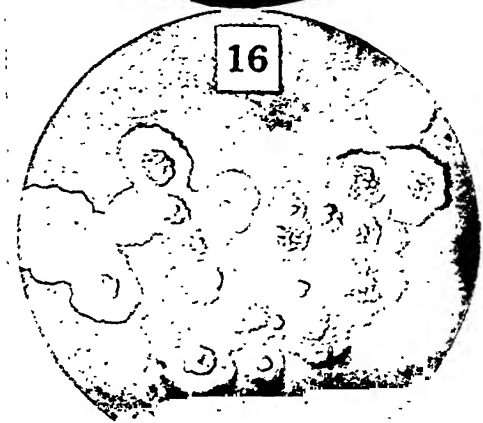
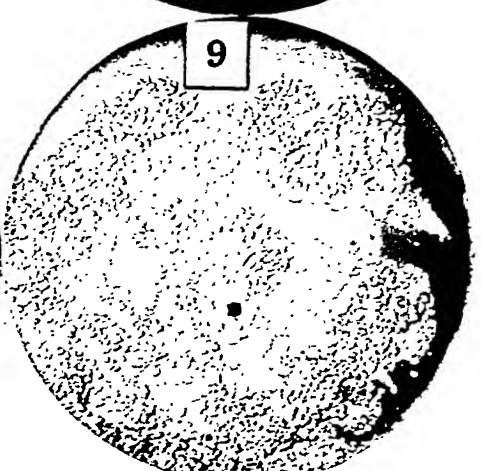
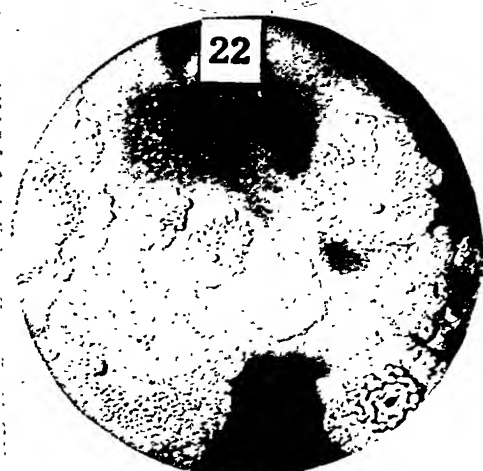
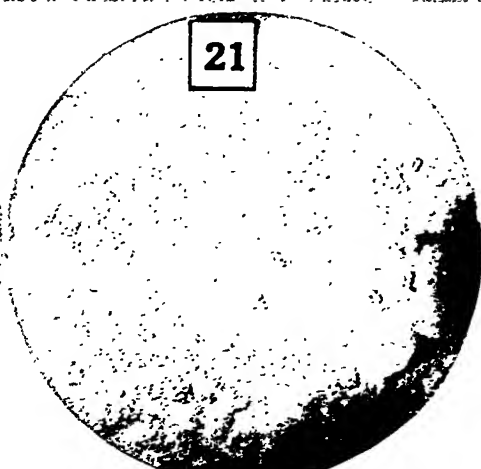
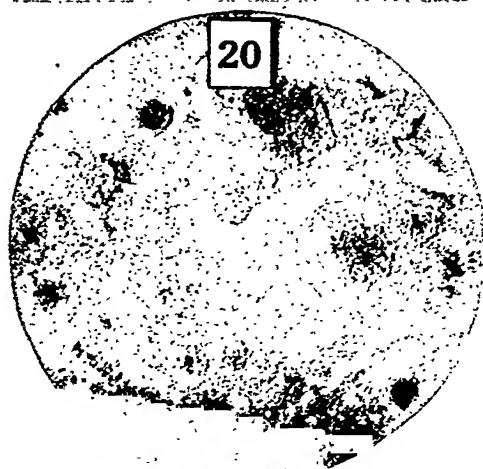




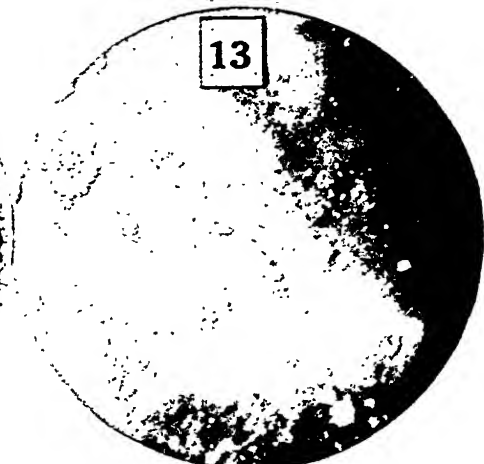
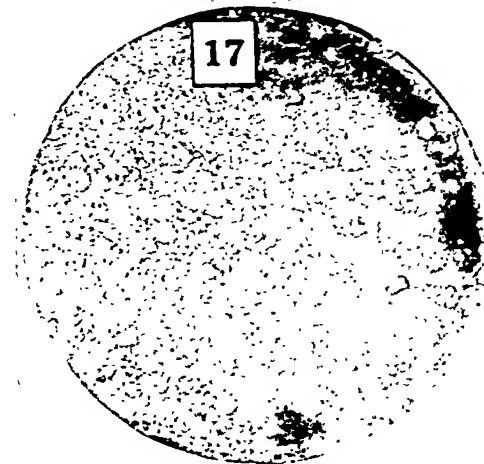
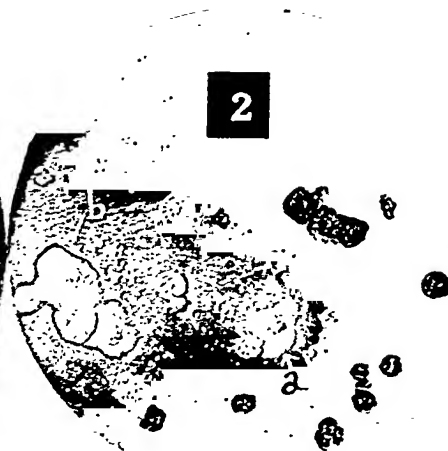
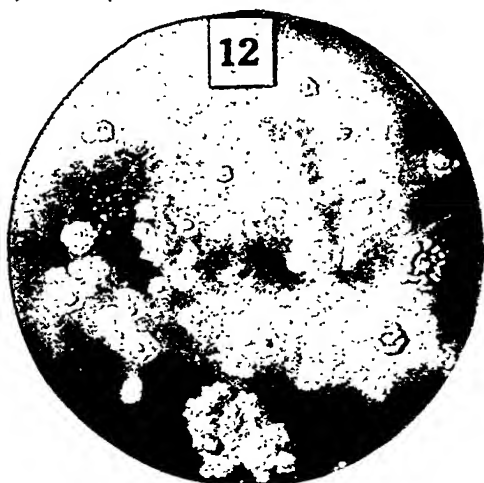


(Petroff and Steenkens: Tubercle bacillus. 1)

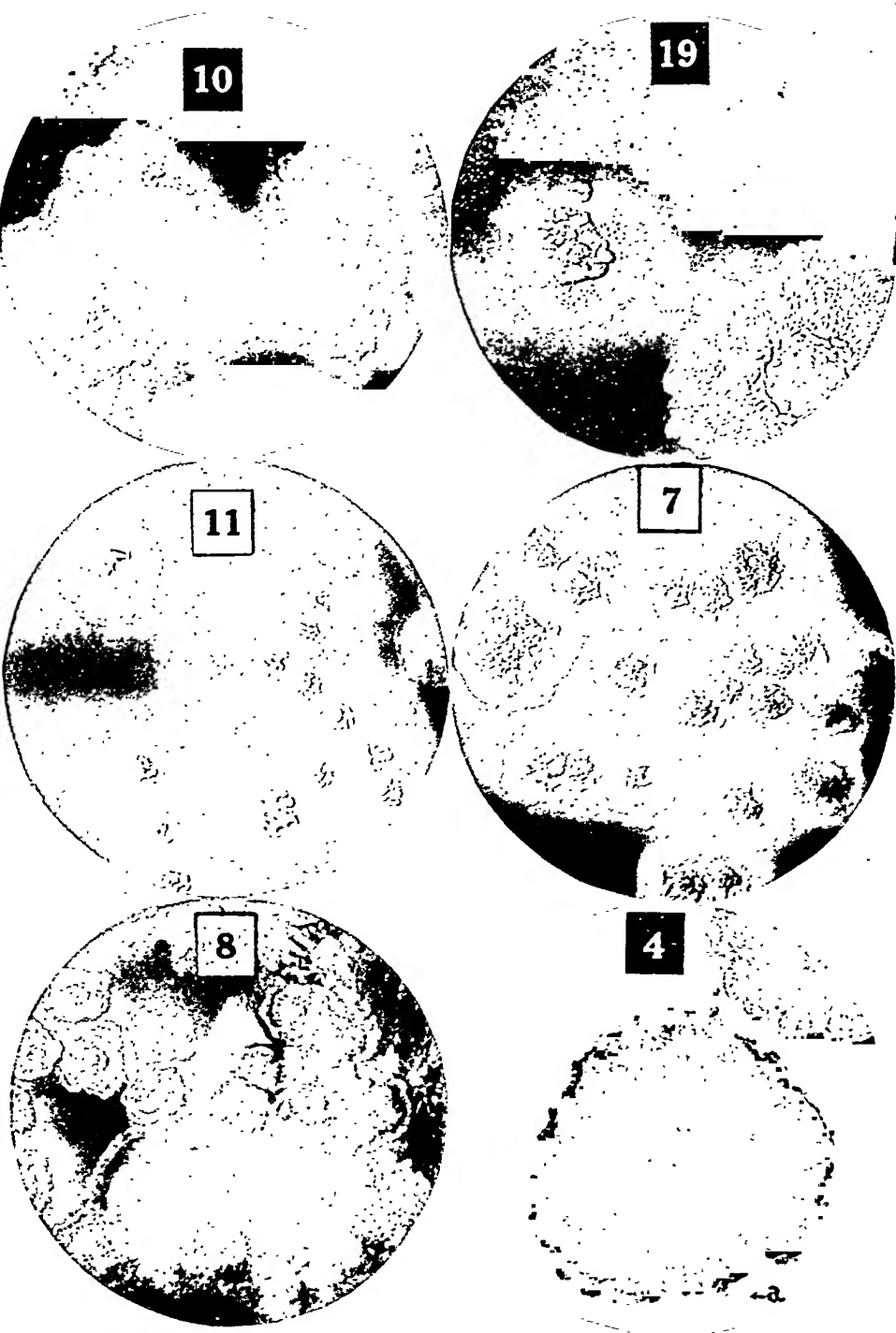






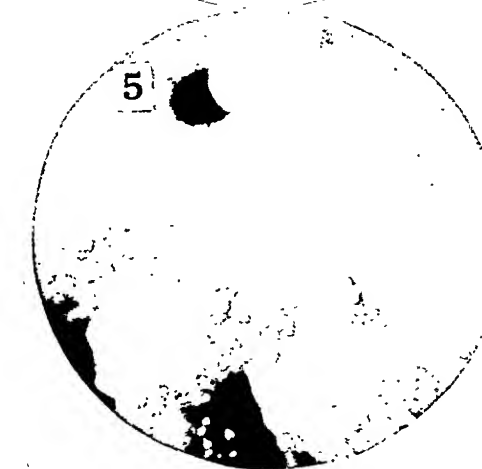
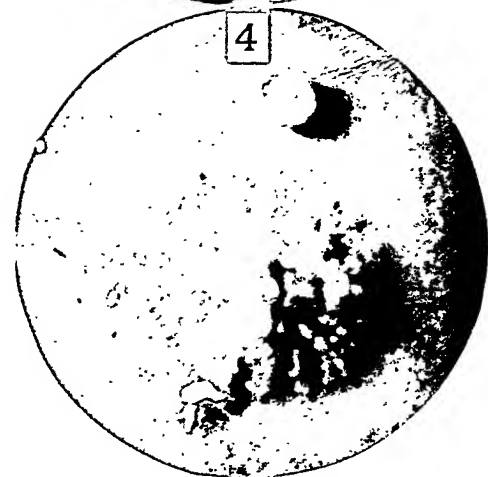
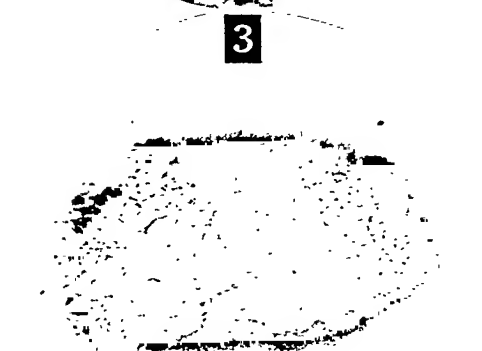
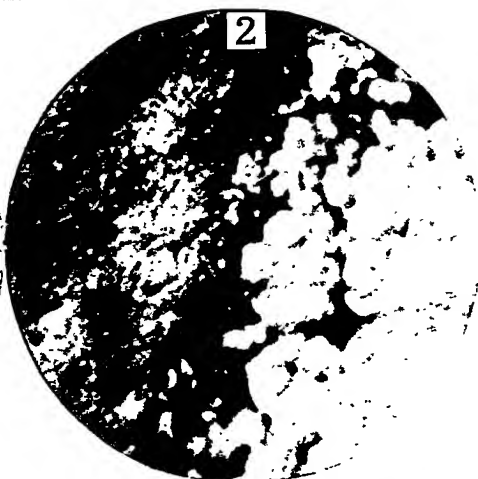
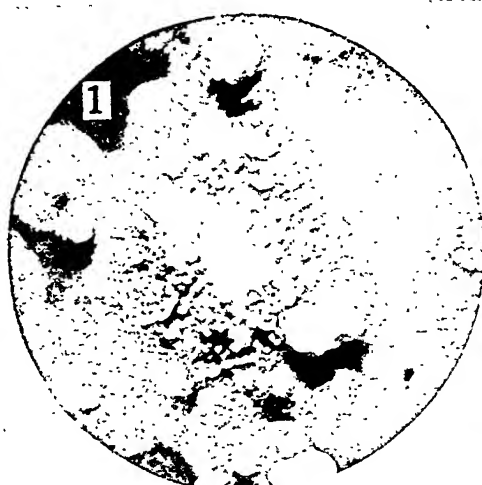














# STUDIES ON MEXICAN TYPHUS FEVER. I

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PLATE 27

(Received for publication, March 10, 1930)

The following notes on experiments with the virus of typhus fever are presented in the briefest possible manner, since to a considerable extent they represent confirmations of previous work. Our results are published because, in a subject as difficult as this one, confirmation of important facts that have been observed a few times only and have in some cases been questioned, constitutes a necessary criterion for the planning of further investigation.

## *The Filtration of Typhus Virus*

There are irregularities in reports of the filtration of this virus. Negative results have been reported by a number of workers (Ricketts and Wilder (1) Anderson and Goldberger (2), Olitsky (3)). Nicolle, Conor and Conseil (4), however, obtained doubtful results, and Nicolle and Lebailly (5) reported two positive filtrations through Chamberland L<sub>2</sub> filters which held back *Bacillus melitensis*.

We carried out a considerable number of filtration experiments through Berkefeld V candles with guinea pig brain proved infectious by control, using the method worked out and published by Ward and Tang (6), which gives almost 100 per cent filtration with brain material of typical filterable agents such as herpes and with the fresh pulp of vaccinia. This, therefore, established an excellent basis for comparison. *Prodigiosus* was used as control, and control inoculations of unfiltered material were always done. This understood, we omit protocols.

Twelve filtrations of brain material from European typhus gave only one filtration in which controls were satisfactory and the virus passed through. Of five



The question has been raised by Nicolle (8) as to whether the virus is present in the leucocytes. This was examined by producing aleuronat exudates in the peritoneal cavities of guinea pigs at a highly infectious stage of the disease, and washing these as free as possible of red blood cells. Guinea pigs injected with washed leucocytes either developed no temperature whatever or came down very much later than those injected with red cells exposed to the same delays and washing manipulation as the leucocytes from the same animal. Leucocyte injected animals occasionally reacted on the sixteenth or seventeenth day after inoculation, while heart's blood of the same animal gave a typical reaction with testicular swelling on the seventh and eighth days. Guinea pigs inoculated with twice washed red blood cells of the same animal reacted like the whole blood inoculated ones. We conclude from these results that the virus is not normally intra-leucocytic, the occasional reactions being probably due to phagocytized virus.<sup>1</sup>

In attempting to wash red blood cells free of virus, we encountered great difficulty. These experiments were carried out by washing with a sufficient amount of broth so that the last supernatant fluid from the red cell washings represented a dilution, in one case, of 1-7000 of the original plasma. It was apparent from these experiments that the virus was either attached to the red blood cells or actually in them, for the cells remained virulent even when four times washed with 50 cc. of broth.

However, in view of the observations reported with Rocky Mountain spotted fever by Spencer and Parker (9), we carried out an experiment in which normal red cells were exposed to infectious plasma and, after standing for 10 minutes, separated and twice washed with broth sufficient to give a final dilution of the infectious plasma of 1-130. The guinea pig injected with the normal cells so treated came down typically with testicular swelling and a temperature of 105°C. on the ninth day, and the virus could be carried on from this animal in passage.

We conclude from these experiments that in Mexican typhus fever, the virus is not normally present in the circulating leucocytes except perhaps occasionally by phagocytosis; that it is not easily separable from the red cells, though it is probably not within them, but adheres firmly to their surfaces; and that it is present in the plasma in a concentration too low to permit the reasonable hope of seeing *Rickettsia* or Mooser bodies in the plasma preparations.

<sup>1</sup>In experiments going on at present we have been able, by the use of a new stain which has been perfected by our associate Dr. Castaneda and is to be described elsewhere, to observe actual phagocytosis of Mooser bodies in polynuclear neutrophile leucocytes.

*Comparative Virulence of Blood Plasma and Tunica Scrapings of the Same Animal*

In a number of experiments of this kind we had irregular results, but on two occasions the tunica material was virulent in much smaller amounts than the blood plasma. On one occasion a dilution of 1-1000 of the material from a whole tunica vaginalis, ground in a mortar with broth, produced the typical disease when the plasma of the same guinea pig did not titrate below 0.1 cc. We have never found plasma infectious in amounts lower than 0.05 cc. A peculiarity of this experiment which we cannot explain is the fact that in the case of the tunica just mentioned guinea pigs receiving 0.1 cc., 0.05 cc. and 0.01 cc. remained negative, whereas the one receiving 0.001 cc. came down. We believe that the comparative titrations indicate that the virus is more concentrated in the tunica where the *Rickettsia* bodies are found. (See also Mooser (7).)

*Tissue Cultures*

Tissue cultures were carried out by the usual manner in guinea pig plasma with tunica cells which had shown the presence of the Mooser bodies. They were incubated at 37.5°, which we believe was a mistake, and the cultures will be repeated at lower temperatures. On the other hand, we were able to observe what was either an increase of the bodies or a plentiful discharge from bursting cells as late as the eleventh day after planting. We were never able to grow these bodies in the second generation, and they disappeared in all tissue cultures when the cells ceased growing.

One week old material from tissue cultures was still infectious. The injection of tissue cultures from which the organisms had disappeared was not infectious.

Our tissue cultures, as far as they have gone, indicate that the *Rickettsia* remain visible only so long as the tissue is alive and growing. The difficulty of transferring to a second generation may be due to the fact that the organisms die out with the cells containing them. Extracellular multiplication could not be proved, because its suggested success—as in the accompanying plate—may easily have been due to a discharge from bursting cells. The tissue cultures seem to indicate again that the observed Mooser bodies or *Rickettsia* are not bacterial in nature, and that the infectiousness of the tissue cultures is roughly parallel with the presence of these appearances.

We were never able to demonstrate the bodies in tissue cultures of spleen or brain.

### *Intraperitoneal Capsules*

Glass capsules, with a hole left in them, were filled with normal plasma and tunica scrapings of infected animals. They were left in the guinea pigs' peritoneal cavities for varying periods. A capsule taken out of the peritoneum of a guinea pig so treated was removed on the sixth day, before the animal had developed any symptoms of disease, and was proved to be still infectious, giving a typical reaction, but no Mooser bodies could be found in smears of the contents. Another guinea pig, into which a similar capsule had been put, showed a temperature of 105° on the twelfth day, but no tunica swelling. The contents of the capsule taken out at this time consisted of clear fluid. There was a fibrinous plug in the mouth of the tube. The smears from these materials were entirely negative for bodies, but the contents injected into another animal gave a typical swelling on the eighth day and a temperature of 105° on the tenth, with typical bodies in the tunica on examination.

Whole infectious blood placed into capsules and similarly observed caused the typical disease, but never showed Mooser bodies when removed and examined.

Our capsule experiments indicate that the virus may remain alive in the peritoneal cavity of a guinea pig in a glass capsule for as long as 12 days, but that the Mooser bodies are too few to be found unless one wishes to assume that all negative investigations of infectious tissues and blood can be explained by the fact that a mutation of form of some kind takes place. This we are loth to assume, largely because of our filtration experiments.

### *Strains of Mexican Typhus Fever without Swelling of the Testicles*

Guinea pigs injected either subcutaneously or intracutaneously do not develop tunica lesions. In such animals a temperature resembling the European typhus temperature develops between the tenth and fifteenth day. If blood from such animals is, at the proper time, injected intraperitoneally into full-grown guinea pigs, these again will often develop swelling. This of course is no proof that we have not carried Mooser bodies along through the negative animals, but it does indicate that the Mooser bodies can remain alive in typical typhus guinea pigs without being detectable in any of the organs, or in the blood, and quite capable of again arousing the characteristic tunica lesion on subsequent proper inoculation.

Whenever, as on three occasions, we have obtained a strain without swelling which has run along for five to seven inoculation generations through adult, intraperitoneally inoculated male guinea pigs, we have found that we were dealing with intercurrent contamination. In our first strain of this kind, which ran for



four generations without swelling, there was no contaminating infection, but the virulence of the strain died down in these animals and was completely gone on the fifth trans-inoculation. The same thing happened in one other strain, and in a third one we found that we were carrying along a low grade bacterial infection, which ended in death in the fourth generation.

In these three cases, the surviving animals were all tested for immunity, and were found to be susceptible.

While such evidence is indirect, it seems to us to support the view that the tunica swelling with Mooser bodies is an integral part of the Mexican typhus infection in guinea pigs, and not an accidental occurrence.

TABLE I

Guinea pig	No. of days between drop in temperature and taking of blood	Protection
1	1	Typical temperature, no tunica lesion*
2	1	Complete protection
3	5	Complete protection
4	5	Incomplete protection typical course**
5	9	Complete protection
6	12	Modified, mild course. No tunica lesion
7	25	No protection
8	34	Typical temperature. No tunica lesion
9	39	No protection

\* This serum, taken on the day the temperature dropped, exerted a very definite modification on the course of the fever, which was late in development and never showed orchitis; but the blood of this guinea pig was still virulent, as proved by inoculation of another animal. There was thus the peculiar condition of a blood already containing protective bodies, but still containing virus.

\*\* This serum was kept about 36 hours in the ice chest before use.

### *Protective Power of Convalescent Serum of Mexican Typhus Guinea Pigs*

Table I shows our results.

Two of these guinea pigs, one completely protected by the 1-day serum, the other completely protected by the 5-day serum, were re-inoculated with virus on the thirty-sixth and fortieth day respectively after the primary mixture had been administered. These animals developed no reaction whatever, while controls were characteristic. It appeared as though the mixture had conferred an active immunity.

These experiments indicate that convalescent serum mixed with virus before injection will protect if the serum is taken between the second and the tenth day after defervescence; that after the tenth day such protective action is doubtful, and that after the twentieth day it is negative. The two animals cited also indicate that guinea pigs so treated may be immune for at least 3 weeks, and that probably—since the injection of serum separately from the virus does not protect—they represent an active rather than a passive immunity.

The rapidity with which the protective bodies disappear from the blood would suggest that they are not of the nature of ordinary bacterial antibodies.

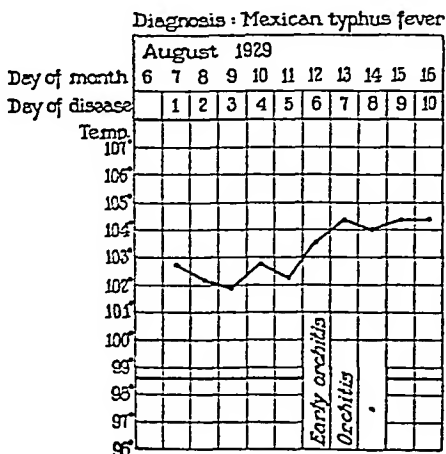


CHART 1.<sup>2</sup> Guinea pig 2-03. Source of virus, simple transfer from 2-02.

Complement fixations were carried out, using virulent plasma as the antigen and protective serum as the antibody, and by such a technique absolutely no indication of complement fixation was obtained.<sup>3</sup>

<sup>2</sup>The word orchitis is used for purposes of convenience; the lesions are almost entirely limited to the tunica and surface of the testicle.

<sup>3</sup>We do not attach much negative value to the complement fixations because of the surely extreme dilution of the antigen in the virulent plasma.

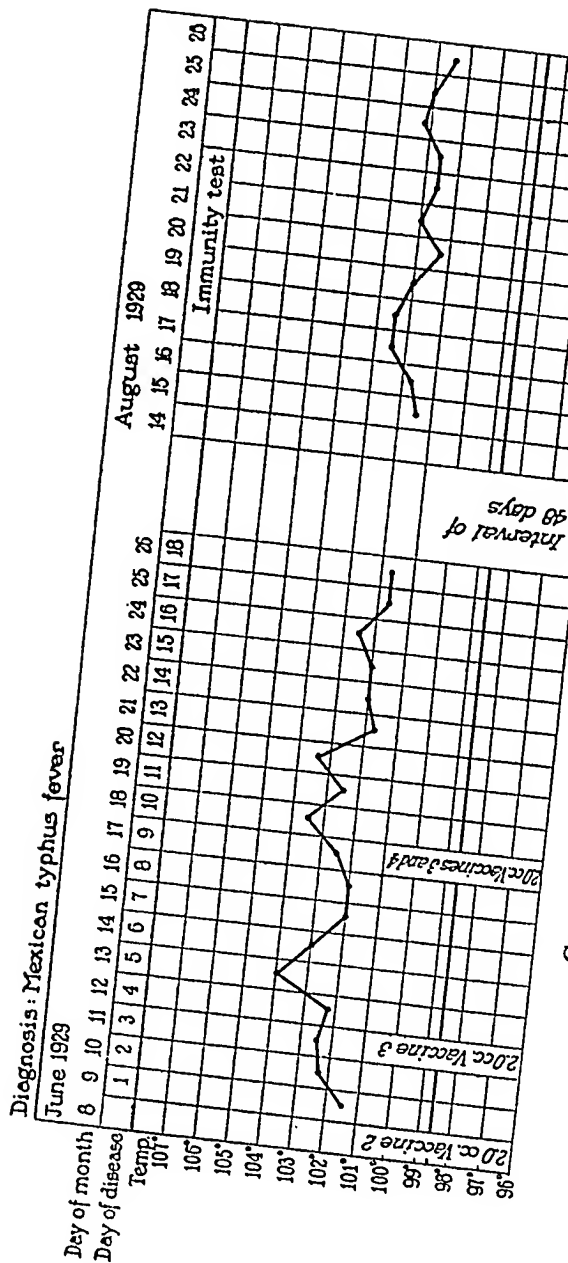


CHART 2. Guinea pig 1-68. Tunic vaccine 2.

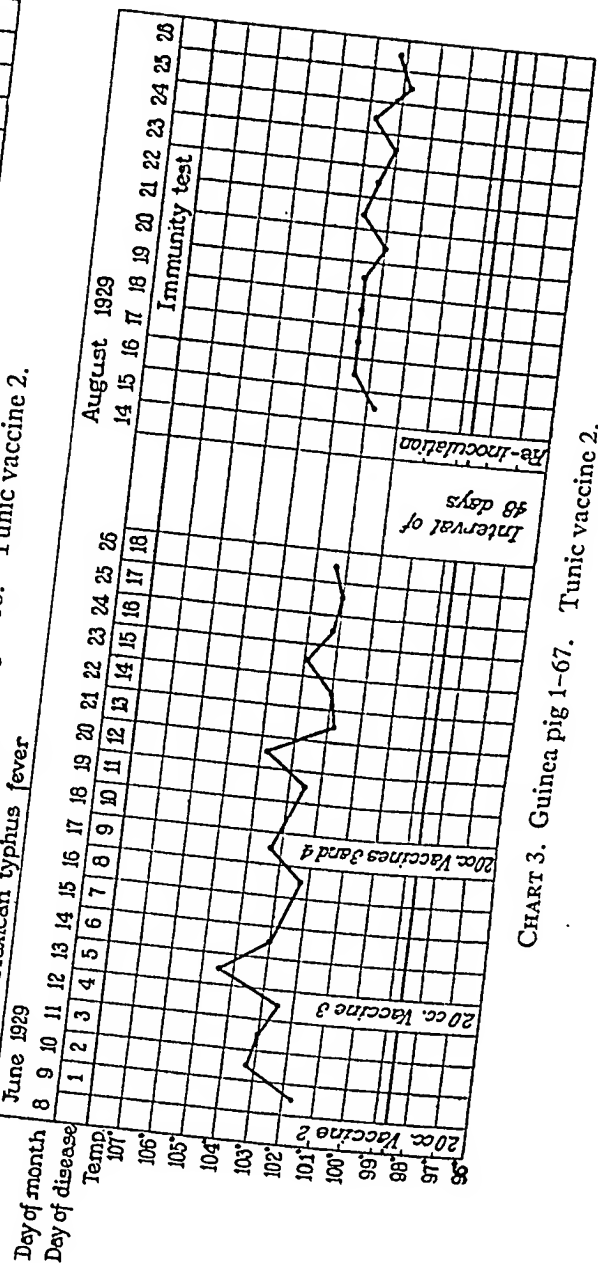


CHART 3. Guinea pig 1-67. Tunic vaccine 2.

*Protection of Animals with Formalinized Tunica Material*

Both from the experiments of Spencer and Parker (9) and of Conner (10) with spotted fever, it appeared that protection with dead infectious material might be feasible in *Rickettsia* diseases, provided that a sufficient concentration of virus could be attained. Having found that the tunica scrapings, containing many of the Mooser bodies, were much more highly infectious than the blood plasma of the same guinea pig, we thought it advisable to attempt active immunization with such material killed with weak formalin solutions by more or less the same method as that which has been successful for distemper in the hands of Laidlaw and Dun-kin (11). Charts 1 to 4 represent a few experiments which gave encouraging

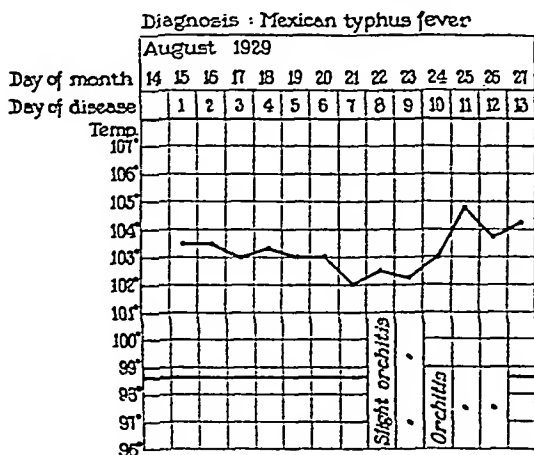


CHART 4. Guinea pig 2-06. Control of reinoculation, simple transfer from 2-03.

results, and which are merely preliminary to a continuation along the same lines with which we are now occupied. The vaccine used was guinea pig tunica taken at a time when it contained Mooser bodies, ground with sand, emulsified in 0.2 per cent formalin, left one night at room temperature and after that put in the ice box. It will be noticed that in the first two animals there was a short rise to 104° after one of the vaccine injections, but that on re-inoculation the animal showed no reaction whatever, although the control came down characteristically.

In the other two, the disease was modified, in that there was swelling of the testes without temperature. This is less significant, because it occasionally—though not often—happens in ordinary Mexican passage animals.

We had one other experiment which was more striking than the first one, but which cannot be included because one of the re-inoculation controls did not react.

Subsequent experiments with formalinized tunics have rendered it uncertain whether we were dealing with dead or attenuated tunic material. This will have to be worked out in the series of experiments with which we are occupied at present, but we believe it worth while to report the above at the present time as an indication that a modification of the disease can be obtained by active immunization with tunic material in which there is a minimum of blood plasma and a maximum of Mooser bodies—a fact which further suggests the etiological importance of these appearances.

### *Human Infection in the Laboratory*

A single case of human infection has occurred in the laboratory. The origin of this case cannot be easily proved, but since the individual was extensively engaged in tunica scraping within the incubation time, and had made only a few blood transfers by heart puncture, which he had been doing for two years without relaxing precautions and without accident, the conclusion is almost forced that this infection originated in tunica material, a small amount of which was probably deposited upon the recently shaven skin of the face.

### SUMMARY AND CONCLUSIONS

The preceding studies on typhus fever, chiefly done with a Mexican strain obtained from Dr. Mooser, concern themselves largely with re-investigations of some of the fundamental problems of this disease.

Filtration experiments carried out with methods almost regularly successful with true filterable viruses, in regard to material, suspension fluid, reaction, nature of filters and pressure employed for filtration, indicate that the virus is not filterable in the ordinary sense in which this expression is employed. It is probably smaller than bacteria and the results of filtration experiments suggest that its magnitude is consistent with the tunica bodies observed by Mooser.

Negative filtrates did not immunize, a result consistent with the previous work of Olitsky.

The virus is present in blood plasma, hardly if at all in leucocytes, and becomes closely associated with the red blood cells, though we do not believe that it is contained in them. It becomes firmly associated

with normal red blood cells when these are exposed to infectious plasma, a result similar to that obtained in Rocky Mountain spotted fever by Spencer and Parker.

In tissue culture, tunica material with Mooser bodies remains alive and virulent for about 10 days, but so far we have not been able to determine that it can keep alive without the presence of living cells. These results do not carry this subject any further than it has been carried for European typhus in tissue cultures with the same method by Wolbach, Schlesinger and Pinkerton (12).

Within glass capsules in the peritoneum of guinea pigs, the virus may remain alive for about the same length of time as in the tissue cultures.

Rough comparative virulence estimations between blood plasma in which it would be hardly possible to find a limited number of Mooser bodies, even though they were present, showed the blood plasma to be less infectious than the tunica material, in which considerable numbers of Mooser bodies were visible.

The testicular swelling characteristic of Mexican typhus and showing the above mentioned bodies—probably *Rickettsia*—may be absent in individual guinea pigs under ordinary conditions and in guinea pigs inoculated by other than the intraperitoneal route. On re-inoculation into the peritoneum after non-orchitic passages, the swelling reappears. Whenever it did not so reappear, we found that the strain had either degenerated in virulence or it had been contaminated by intercurrent infection. Though we can not prove it at the present time, we believe that the tunica lesion is an integral part of this disease in guinea pigs, and not an accidental accompaniment.

Convalescent blood from Mexican typhus guinea pigs mixed in the test tube with virus affords protection if the blood is taken between the first to the tenth day after defervescence. After the third week, the blood no longer contains protective bodies although the guinea pigs may still be immune.

In one case a serum was obtained which was both protective in such a test but at the same time seemed still to contain virus, a result which we cannot explain.

No complement-fixing antibodies were found when virus serum was used as antigen and convalescent serum as antibody. The low concentration of the virus in the serum may account for this.

In a limited number of observations guinea pigs which were negatively inoculated with virus-serum mixtures proved on re-inoculation to be immune. In one of these cases the protective serum mixture with the virus was taken 1 day, in the other 5 days after temperature had returned to normal and the re-inoculations were done 36 and 40 days after the primary injection. This recalls similar experiences of Nicolle and encourages further immunological study in this direction.

In a number of experiments active immunization with formalinized tunica material containing large numbers of the Mooser bodies seems to have modified the course of subsequent inoculations in the direction of protection.

A single accidental human infection seemed particularly associated with tunica material, although this cannot be positively asserted.

All that part of our work which has bearing on the infectious agent is consistent with the assumption that the small, Giemsa-staining bodies observed by Mooser in the tunica of Mexican typhus guinea pigs represent the virus of the disease.

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#### EXPLANATION OF PLATE 27

FIG. 1. Mooser bodies (*Rickettsia*) in an 8 day tunica tissue culture.

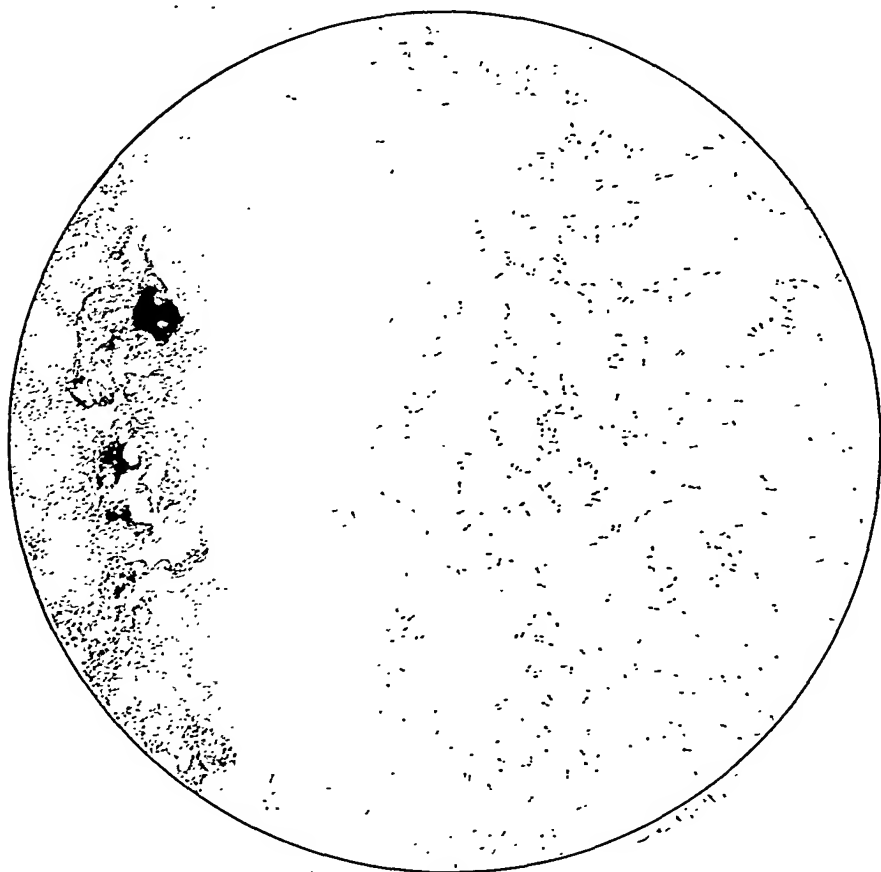


FIG. 1

(Zinsser and Batchelder: Mexican typhus fever. 1)





# CENTRIFUGE EXPERIMENTS WITH THE VIRUS OF VACCINIA

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The nature of filtrable viruses is still a controversial subject. Gordon (1) suggested that rabbit vaccinia virus is associated in particles big enough to be thrown down by centrifugal force and even thought that the virus might come down under the action of gravity. MacCallum and Oppenheimer (2) stated that vaccine granules can be freed from lymph by differential centrifugation. Bedson (3) supported his theory that the non-passage of herpes virus through a colloidal membrane was due to the size of the virus by the fact that the virus in suspension can be concentrated by high-speed centrifugation. More recently Bland (4) reported similar results in his filtration and centrifuge experiments with vaccinia virus. These results have been criticized, however, on the ground that the experiments were conducted with emulsions or diffusates, where the virus might have been adsorbed by contaminating microorganisms or by cellular structures, and that consequently, when the adsorbing material was centrifuged down, it carried the virus with it.

In a previous communication (5) we have reported that if tissue containing the virus of herpes or vaccinia was suspended in hormone broth instead of saline solution, active filtrates could be constantly obtained. We suggested that if careful centrifuge experiments were carried out with cell-free filtrates, it might be possible to throw some light upon the real nature of the virus. The work recorded here is the result of such an investigation carried out with vaccinia virus.

## *Preparation and Testing of the Virus Filtrates*

Active filtrates of vaccinia virus were prepared according to the method previously described, except that ordinary powdered Japanese glass was used instead

of sand. Fresh virus containing tissue was thoroughly ground in a mortar with the glass powder, and a 5 per cent suspension was made in hormone broth. It was then centrifuged for half an hour at about 2000 r.p.m. and the supernatant fluid poured off for filtration. A Berkefeld V filter was employed after testing its water flow, and a new filter was used for each filtration. The filtration was carried out as soon as possible after the tissue was taken from the animal and always under a negative pressure of 50 cm. of mercury. The filter was further controlled by adding a little of a culture of *B. prodigiosus* to the suspension before filtration was commenced. Big, albino rabbits were used for titrating the activity of the filtrates. The back of the animals was shaved the previous day and the points of injection

TABLE I

Dilution	Unfiltered control			Filtrate		
	3rd day	5th day	7th day	3rd day	5th day	7th day
Undiluted	Not tested	Not tested	Not tested	++	+++	++++
1:10	++	*++++	++++	+	++	+++
1:50	++	++++	++++	—	++	++
1:100	+	++++	++++	—	++	++
1:300	+	++++	++++	—	++	++
1:500	+	++++	++++	—	++	++
1:800	±	++++	++++	—	+	++
1:1000	—	++++	++++	—	—	+
1:1500	—	++++	++++	—	—	—
1:2000	—	+++	+++	—	—	—
1:3000	—	+++	+++	—	—	—
1:5000	—	++	++	—	—	—

\* In this and the following tables + + + + represents a maximum reaction and ± a minute papule.

were marked out with a circular ink stamp. The injections were made intradermally, and 0.05 cc. of each dilution was injected. The titration of one such preparation prepared from virus pulp S. M. C., Source 683, is given in Table I.

### *Centrifuge Experiments*

5 or 10 cc. of the filtrate was centrifuged in a conical centrifuge tube in an International size 1, Type S. B. centrifuge for varying lengths of time at the maximum speed—about 4000 r.p.m. Then 0.1 cc. of the fluid was carefully pipetted from the surface and the remaining fluid discarded until a similar amount was left in the bottom of the tube. Dilutions were made in hormone broth of both upper and lower fractions as well as of the uncentrifuged filtrate which served as the control. The dilutions were then injected intradermally into a rabbit. The

result of a 2 hour centrifuge experiment conducted with a green virus filtrate is shown in Table II.

As fresh calf pulp was not always easily obtained, an attempt was made to use rabbits as a source of supply. The preparation of rabbit green virus was very simple; the animals were inoculated on the skin

TABLE II

Dilution	Bottom fluid	Surface fluid	Control
Undiluted	++++	±	++++
1:10	++++	—	+++
1:50	+++	—	++
1:100	++++	—	++
1:300	++	—	++
1:500	+++	—	++
1:800	++	—	++
1:1000	++	—	±
1:1500	++	—	—

In this and the following tables readings were taken on the 5th day, unless stated otherwise.

TABLE III

Dilution	Surface fluid	Bottom fluid
Undiluted	+	++++
1:10	—	++++
1:100	—	++++
1:200	—	+++
1:400	—	+++
1:600	—	++
1:800	—	++
1:1000	—	++
1:1200	—	++

and the virus collected on the fifth day. Table III shows the result of a centrifuge experiment with a filtrate prepared from rabbit pulp. The end-point of the activity of the uncentrifuged filtrate was a dilution of 1:800.

Three strains of neurovaccine derived from intracerebral inoculation of different filtrates were obtained. From these 3 neurotropic strains

a number of active filtrates were prepared. Table IV gives the result of one of the centrifuge experiments made with a filtrate prepared from a neurovaccine brain.

All these experiments show a definite concentration of the virus in the lowermost layer of the centrifuged fluid, leaving the supernatant portion practically inactive. Evidence then seemed to be in favor of the theory that the virus itself or at least its aggregates might really be associated in particles big enough to be spun down by centrifugal force because of the fact that the filtrates contained no microorganisms or other cellular elements upon which the virus might be adsorbed, and so carried down along with this adventitious matter. It has been suggested (6), however, that inert particles present in the filtrates

TABLE IV

Dilution	Surface fluid	Bottom fluid	Control
1:50	—	+++	++
1:100	—	++	++
1:300	—	++	++
1:500	—	++	++
1:800	—	+	+
1:1000	—	++	±

might act as adsorbents, and carry down the virus. In order to meet this criticism, some further experiments were carried out.

The separation of coarser from finer particles by fractional centrifugation is an ordinary laboratory procedure. Since the inert particles assumed to be present in the broth filtrates must be bigger and heavier than the virus, their separation by fractional centrifugation should be possible.

In order to carry out such a separation, more active filtrates are necessary. Such filtrates have been prepared by Ward (7). The only difference in his technique from that already described in this paper is the substitution of pyrex glass powder for ordinary glass powder or sand as a grinding material. Table V gives the titration of a calf green virus filtrate prepared by this modified technique from virus pulp S. M. C., Source 723, and a neurovaccine Filtrate 084 obtained from a rabbit brain originally obtained from vaccine lymph, Lot 97, National Epidemic Prevention Bureau.

TABLE V

Dilution	Green virus 723		Neurovaccine 084	
	Filtrate	Unfiltered	Filtrate	Unfiltered
1:10	+++	++++	++++	++++
1:50	+++	++++	+++	++++
1:100	+++	+++	+++	++++
1:300	+++	+++	+++	++++
1:500	+++	+++	+++	++++
1:800	++	++	+++	+++
1:1000	+	++	++	+++
1:1500	++	++	++	+++
1:3000	++	+++	++	++
1:6000	++	++	++	++
1:12000	++	++	++	++
1:24000	++	++	+	++
1:48000	++	+		
1:120000	±	+		
1:600000		+		
1:1200000		+		

TABLE VI

Dilution	Control	Surface fluid	Bottom fluid
1:10	+++	+++	++++
1:50	++	+++	++++
1:100	++	++	+++
1:300	+++	+++	++++
1:500	+++	++	++++
1:800	++	++	++++
1:1000	++	++	+++
1:1500	++	+	+++
1:3000	+	+	+++
1:6000	+	+	++
1:12000	±	—	++
1:24000	±	—	++
1:48000	—	—	++
1:120000	—	—	++
1:600000	—	—	+
1:1200000	—	—	+

*Fractional Centrifuge Experiments*

10 cc. of calf green virus Filtrate 723 was centrifuged for 1 hour at 3500 r.p.m. in a centrifuge tube stoppered with a rubber cap. Then 5 cc. from the surface was carefully pipetted off without disturbing the lower portion and transferred to a second tube which was centrifuged for 4 hours at the same speed. Surface and bottom specimens were collected and then the various layers were mixed and a third specimen taken to serve as control. All 3 specimens were then titrated on the same rabbit. The readings were taken on the third day. Table VI shows the result of this titration.

Another experiment was then done in which Filtrate 723 was centrifuged for 4 hours at 3500 r.p.m., the upper half of the fluid removed and placed in the refrigerator over night. Next morning this was centrifuged for 4 hours at the same

TABLE VII

Dilution	Surface fluid	Bottom fluid	Control
1:10	+	++	++
1:50	—	++	+
1:100	—	++	+
1:300	—	++	±
1:500	—	+	+
1:800	—	+	—
1:1000	—	+	—
1:1500	—	+	—
1:3000	—	—	—
1:6000	—	—	—

speed. Table VII shows the result of the titration of the upper and lower layers as well as of the whole fluid as control. Readings were taken on the third day.

From the results of Tables VI and VII, especially from Table VII, it seems improbable that the concentration of the virus was due to the adsorption of the virus by the inert particles in the filtrates, because such particles must have been thrown down by the preliminary centrifugation. The results shown in Table VII were confirmed by several other experiments in which the first centrifugation was carried out at 3500 r.p.m. for 4 hours.

## DISCUSSION

The ease of filtration of vaccine virus through diatomaceous filters has been demonstrated once more. The success of filtration depends

of course upon many conditions, but the material used for grinding is evidently an important one. Ward's observation (7) that pyrex glass powder serves this purpose better than anything else has been confirmed, and it has been noted that ordinary glass powder is no better than sand. The superiority of pyrex fragments for this purpose is probably due to their not adsorbing the virus rather than to their sharper edges.

Another important condition necessary is the use of very fresh tissue containing the virus. Filtrates prepared from tissue removed from the animal for some time before being filtered are often quite inactive or only slightly active. It is possible that the size of the virus may be altered somehow by some unknown change taking place in the cellular substance of the tissue after death. Ward (7) has shown that if oxygen is excluded from the tissue after removal from the animal, very active filtrates can be obtained after some days, whereas the filtrates of tissue exposed to the air for the same length of time are inactive.

With regard to the success of the centrifuge experiments in concentrating the virus, in the author's opinion it is due to the corpuscular nature of the virus or its aggregates rather than to the presence of inert particles adsorbing and carrying down the virus, for these particles should have been removed by the prolonged preliminary centrifugation.

#### SUMMARY

Centrifuge experiments have been carried out with cell-free, active filtrates of vaccinia virus. The experiments have shown that the virus can be concentrated by this method, even in filtrates which have been subjected to prolonged preliminary centrifugation to throw down any inert particles which may have been present in the original filtrate. This fact, together with the knowledge that the virus can be almost completely held back by the Berkefeld N filter, as reported previously (5) indicates that the virus may be of considerable size.

The writer wishes to express his gratitude to Dr. R. C. Robertson of the Health Department of Shanghai Municipal Council for many specimens of fresh virus pulp.



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# TOTAL WATER AND CHLORIDE CONTENT OF DEHYDRATED RATS

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## INTRODUCTION

Dehydration of the body as produced experimentally by placing an obstruction at the pylorus has been extensively studied from the point of view of the changes in the composition and physical properties of the blood caused by the continued loss into the stomach of water and materials; chiefly sodium and, in larger quantity, chloride ion (1, 2, 3, 4). As regards the actual extent of withdrawal of water and of these substances, Gamble and McIver have shown that in rabbits several times the initial blood plasma content of water, of sodium and of chloride ion enters the stomach during the survival period following an experimental obstruction at the pylorus (5). It is thus evident that stores of water and materials behind the vascular system are extensively drawn upon in support of blood plasma volume and composition. The purpose of the study, the results of which are here presented, was to measure the degree of depletion of the water and chloride ion content of the body as a whole which may result from upper intestinal obstruction. It would have been desirable to have determined also the amount of sodium remaining in the body but this was not attempted. Water and chloride ion were measured in the skin of the animal and in the remainder of the carcass, after removing the gastro-intestinal tract, by the method described below. An essential point regarding these measurements is that they were obtained by drying and then digesting the entire skin or carcass, that is, the material was used *in toto*. In some instances the animal was exsanguinated as completely as possible and the water and chloride ion content of the blood determined.

The initial experimental procedure was obstruction of the pylorus by ligature. The resultant continued loss of gastric secretions will

obviously cause body fluid depletion. On reflection, however, it is apparent that the situation contains other factors which may also be expected to cause reduction of the total water content of the body. Pyloric obstruction produces, besides a loss of gastric secretions, the circumstances of fasting and of complete water deprivation. As a result of fasting there must occur consumption of body fat, glycogen and, to some extent, of body protoplasm, with release of constituent water. It would also seem probable that the circumstance of water deprivation will tend to cause subsidence of the water content of the body. With the purpose of appraising together these two additional factors of water removal which are incidentally produced by upper intestinal obstruction, measurements of the loss of water and of chloride ion by unoperated animals subjected to a period of fasting and water deprivation were obtained.

Another item in the plan of study was comparison of the loss of water and chloride ion by rats fasted and deprived of water with the losses found for rats following an experimental obstruction placed at the lower end of the small intestine, water and food being again withheld. The purpose here was to test the expectation that lower intestinal obstruction, in contrast with pyloric obstruction, is a small or perhaps a negligible cause of dehydration, because of a probably more or less complete reabsorption of gastro-intestinal secretions by the small intestine.

### *Procedures*

*Operative.*—The animals used for these experiments were young adult white rats. Under ether anesthesia the abdomen was opened and obstruction established at the pylorus or just above the appendix by tying with strong silk thread. The incision was closed and covered with collodion. The rats recovered very quickly from the operation. After the desired interval they were killed by ether, the skin was rapidly removed and the gastro-intestinal tract, clamped at both ends, was dissected from the remainder of the carcass. Skin and carcass were then placed in the weighed flasks of the digestion apparatus. When blood was withdrawn this was done under ether anesthesia by opening the thorax with a U-shaped incision after reflecting the skin and delivering the heart through this incision into the mouth of a weighing bottle and then opening the ventricles by snips with a small scissors.

*Analytical.*—The flask containing the fresh material was weighed and then placed in an electric oven at 100°C. until a constant dried weight was obtained.

A measured excess of  $N/20$  silver nitrate solution was then placed in the flask, several drops of caprylic alcohol added and the reflux condenser fitted on, the joint being of ground glass. 100 cc. of nitric acid was then added through the condenser tube and the mixture cautiously heated over an electric plate until foaming had ceased. The electric heater was then turned full on. Occasionally caprylic alcohol was required during the process of digestion. After a period of 1 or 2 hours, digestion was complete except for tissue fat which was found to have separated completely with the formation of large globules on the top of the mixture. The condenser tube was then rinsed, the flask stoppered and placed in the ice box until the fat solidified. The mixture was then filtered into a volumetric flask, the original flask and precipitate being thoroughly washed with distilled water. The filtrate was then made up to volume. Four 10 cc. samples of the filtrate were pipetted into large pyrex test tubes and titration carried out with  $N/40$  ammonium thiocyanate. One sample was titrated to the first visible end point and another was not carried to the end point. These two tubes were used as comparators in titrating the two remaining samples. Titration figures checked very accurately. Calculation: cc.  $N/20$  silver nitrate added originally less one-half of the amount of  $N/40$  thiocyanate required divided by 20 gives milli-equivalents of chloride ion in the sample.

#### RESULTS

The data obtained from a control rat, from a rat deprived of food and water for 18 hours, and from three rats sacrificed 12 hours after obstructing the pylorus, are presented in Table 1. It should be noted that the average weight of the obstructed rats (192 gm.) is a bit greater than the weight of the control animal (186 gm.) and that in the food and water deprivation experiment the period was 18 hours whereas the interval following pyloric obstruction was 12 hours. The longer period of fasting and water deprivation was used on the basis of an incorrect expectation that these circumstances would be relatively small factors of dehydration. The survival period following pyloric obstruction was found by preliminary experimentation to be between 30 and 40 hours, so that the data in the table describe changes occurring during the first third of the survival period. This short interval was chosen because it had been found that after 12 hours there occurred loss of stomach contents by vomiting or regurgitation and it was desired to compare the amount of chloride ion found in the stomach with the measurement of loss from the body. As regards the total quantity of blood collected by the technic described above, we have considerable doubt that it dependably measures blood volume. In the case of the

obstructed rats, there was evident increase in viscosity which very probably interfered with complete collection. The data are included in the table in order to complete the total values for body weight, water and chloride. The measurements of water per cent and of chloride ion concentration are, of course, not disturbed by the probable error in volume.

In spite of these appreciable defects in the plan of study, comparison of the several sets of data in the table produces certain quite evident

*Showing Losses of Water and of Chloride Ion Produced*

Losses are estimated by subtracting the values found from those established by the control skin and the gastro-intestinal tract. The "Total" values are the sum of those found for skin

Experimental procedure	Weight		Skin					Wt.
	Initial	Final	Wt.	H <sub>2</sub> O	H <sub>2</sub> O	Cl	(Cl)	
	gm.	gm.	gm.	gm.	per cent	m.eq.	M	gm.
Control.....	186		33.9	18.6	54.9	2.26	.121	129
Fasting and water deprivation 18 hrs.....	185	172	28.3	15.9	56.2	1.89	.119	119
Loss, absolute.....		13	5.6	2.7		.37		10
Loss, per cent.....		7%	16.5%	14.5%		16.4%		7.8%
Pyloric obstruction 12 hrs.	191	180	28.1	13.9	49.5	1.48	.107	117
	192	180	26.1	14.3	54.8	1.46	.102	120
	193	182	27.3	14.2	52.0	1.51	.106	121
Averages.....	192	181	27.2	14.1	52.0	1.48	.105	119
Loss, absolute.....		11	6.7	4.5		.78		10
Loss, per cent.....		6%	19.7%	24.2%		34.5%		7.8%

findings which may be briefly discussed. To consider first the effects of fasting and water deprivation: we note that the 18 hour period reduced the weight of the animal from 185 gm. to 172 gm. As regards the composition of this loss in terms of water and solids, information is provided by comparing the "total" values for the control and for the fasted animal obtained by adding together the measurements for skin, tissues and blood. A closely parallel loss of water and of solids is indicated by the fact that the values for body weight loss and for reduc-

tion of water content are 8.4 per cent and 8.1 per cent respectively. The loss of weight is thus, as would be expected, the result of consumption of body substance *in toto* rather than of dehydration. Taking the skin by itself, however, and comparing the per cent loss of weight and of water it is unexpectedly evident that a larger loss of solids than of water has occurred producing an appreciable increase of water content. In the remainder of the carcass, designated "tissues" in the table, the loss of water slightly exceeds the loss of solids. The total

*Water Deprivation, and (2) by Pyloric Obstruction*

material designated "Tissues" was the remainder of the carcass after removing blood,

(Cl)	Blood					Total					Gastric contents	
	Wt.	H <sub>2</sub> O	H <sub>2</sub> O	Cl	(Cl)	Wt.	H <sub>2</sub> O	H <sub>2</sub> O	Cl	(Cl)	H <sub>2</sub> O	Cl
<i>M</i>	<i>gm.</i>	<i>gm.</i>	<i>per cent</i>	<i>m-eq.</i>	<i>M</i>	<i>gm</i>	<i>gm.</i>	<i>per cent</i>	<i>m-eq.</i>		<i>gm.</i>	<i>m-eq.</i>
.050	4.52	3.64	80.6	.347	.095	167	111	66.4	7.03	.064	0	0
.046	5.90	4.60	78.0	.437	.095	153	102	66.7	6.06	.060	0	0
						14	9		.97			
						8.4%	8.1%		13.8%			
.042	3.54	2.68	75.7	.221	.082	149	95	63.8	4.95	.052	15.9	2.32
.044	2.53	1.96	77.5	.175	.089	149	99	66.4	5.27	.053	15.0	2.27
.040	2.06	1.51	73.3	.122	.081	150	97	64.7	4.91	.051	15.9	2.36
.042						149	97	65.0	5.04	.052	15.6	2.32
						18	14		1.99			
						10.8%	12.6%		28.3%			

chloride loss produced by fasting and water deprivation is 13.8 per cent of the initial body content, and roughly one-third of this, and also of the water loss, is derived from the skin. The per cent losses from the skin are, it may be noted, about twice as great as those from the tissues.

When, added to the circumstances of fasting and water deprivation, a loss of gastric secretions is produced by placing an obstruction at the pylorus, body weight falls in 12 hours from 192 gm. to 181 gm. (average for three animals). But inasmuch as the final weight (181 gm.)

cludes the gastric secretions trapped in the stomach, the 11 gm. of body weight loss here observed is presumably referable to the factors of fasting and water deprivation and agrees roughly with the loss of 13 gm. produced in the preceding experiment by these circumstances, in the absence of obstruction. On opening the abdomen the stomach was found enormously distended and to contain water to the surprising extent of 15.6 cc. (average of findings). If 15.6 gm. be added to the observed body weight loss, 11 gm., a total weight loss of 26.6 gm. is obtained. The estimated weight loss from skin, tissues, and blood taken together given in the table is 18 gm. The considerable discrepancy between these two data is, we believe, explained by absorption of water from the gastro-intestinal tract below the obstruction. Unfortunately weight measurements of the tract were not made but it was observed *post mortem* that, in striking contrast to the enormous stomach, the remainder of the gastro-intestinal tract was completely empty and shrunken to a tiny cord. The inference thus produced is that about 8 or 9 cc. of the water lost into the stomach is replaced by water absorbed from the intestines. This source of body fluid replenishment of course, soon exhausted but is here seen to be of an extent sufficient to cover about one-half of the water loss into the stomach during the first 12 hours following obstruction. Although the effect of the loss of gastric secretions on the water content of the body is thus greatly reduced, the estimations of net loss of weight and of water, 10.8 per cent and 12.6 per cent respectively, indicate an appreciably more extensive loss of water than of substances, and this is found to be true in terms of per cent water content for both skin and tissues. The chloride loss is, as would be expected, greatly increased as compared with the loss found for fasting and water deprivation without obstruction, being nearly one-third of the initial chloride content of the body, and is completely accounted for by the amount found in the stomach. An extensive fall of chloride ion concentration is found in the skin and a considerable decrease occurs also in the blood, changes obviously referable to the much higher concentration of chloride ion in the gastric secretions than in body fluids.

These data enable us to appraise the two factors of water removal from the body in the presence of upper intestinal obstruction; (1) water leaving the body as a "waste product" of the consumption of

protoplasm due to the circumstance of fasting and, (2) water lost into the stomach because of the failure of reabsorption of gastric secretions. According to the results of these experiments the first factor is of the same magnitude as would be produced by fasting and water deprivation in the absence of obstruction and when obstruction is present it accounts for more than half of the water loss over the experimental period here studied. The large size of this factor was to us a surprising finding. The data for skin and for tissues given in Table 1 are graphically presented in Fig. 1, Sections A and B, with the purpose of bringing out this point more clearly. It can here be appreciated that although loss of gastric secretions considerably accelerates the decline of the values measured, fasting with water deprivation is the larger factor in the situation.

As regards the loss of water due to the second factor, that is, to failure of reabsorption of the gastric secretions, there remains for consideration the interesting question of its source. It has been argued that this water derives entirely from interstitial body fluids (6). The data here given are not definitely informative on this point. Since the skin, because of its loosely areolar understructure, serves as a chief depot of interstitial fluid, a relatively much larger loss of water from the skin than from other body tissues would be expected. This was the case in these experiments. It may be computed from the data in Table 1 that three-fourths of the water loss referable to obstruction derives from the skin. The small remainder comes from tissues which, although they contain relatively less interstitial water, are in bulk more than twice that of the skin. It would therefore seem possible that the loss of water from the tissues referable to obstruction, which, as may be seen in the table, causes a just measurable reduction of per cent water content, is entirely composed of interstitial water. Unpublished data obtained by one of us (McKhann) bearing on this point may appropriately be inserted here. They consist in measurements of per cent water content of two parenchymatous tissues, heart muscle and brain, taken from rats dehydrated by repeated intraperitoneal injections of hypertonic (4.5 per cent) sodium chloride solution and are presented in Table 2. They show, especially in the case of brain tissue, a remarkably successful defense of the normal water content.

Data from another experiment measuring the changes found over a



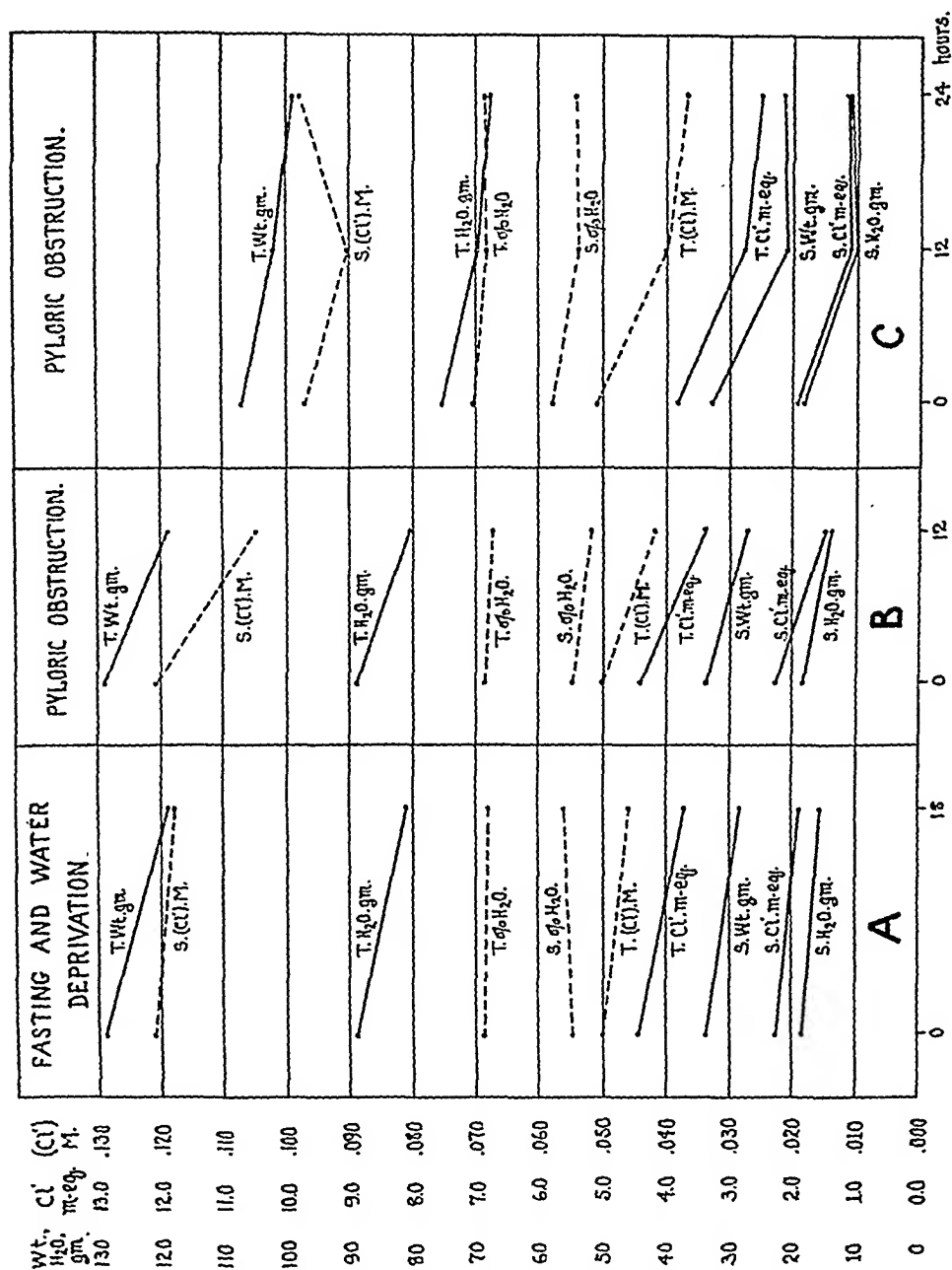


FIG. 1. Sections A and B present graphically the measurements of body weight, water, and chloride ion for skin (S) and for tissues (T) given in Table 1. The data plotted in Section C are from another series of animals and represent the effects of pyloric obstruction over a longer period (24 hours).

longer interval following pyloric obstruction are recorded in Section C of Fig. 1. These are from three rats of uniform weight (155 gm.). Striking decline in the rate of loss of water and chloride ion during the second 12 hours following obstruction is evident. Indeed, in the case of the skin, there is, according to the data, no further depletion of water and materials after the first 12 hours. Apparently by then the subcutaneous depots are completely exhausted and further losses must be entirely sustained by the other tissues. An extremely slight depression of the per cent water content of the tissues is again found.

In the first section of Fig. 2 are plotted measurements descriptive of the effects of prolonged fasting and water deprivation. After the

TABLE 2

*Per Cent Water Content of Kidneys and Brains from Rats Dehydrated by Repeated Intra-Peritoneal Injections of Hypertonic Sodium Chloride Solution*

Body weight, gms.		Water content, per cent	
Initial	Final	Kidneys	Brain
350	280	76.2	78.8
347	298	75.5	77.1
298	242	78.8	78.5
342	258	75.2	78.6
263	185	76.8	78.8
Averages of control data.....		77.2	78.5

initial 18 hour period a much less rapid rate of loss of body weight, water and chloride ion is established. The entire survival period was 187 hours. The total losses in per cent of initial values were as follows: body weight 39.6 per cent, water 38.3 per cent, chloride ion 35.3 per cent. A curious finding shown in the figure is the extensive rise of chloride ion concentration in the skin and the same change, though of much less degree, in the tissues. The per cent water content of the tissues remains nearly stationary, exhibiting a slight trend toward increment rather than depletion. In the skin an increase in per cent water content occurs more appreciably. In the experiment which produced the data given in the second section of Fig. 2, in addition to fasting and water deprivation, an obstruction

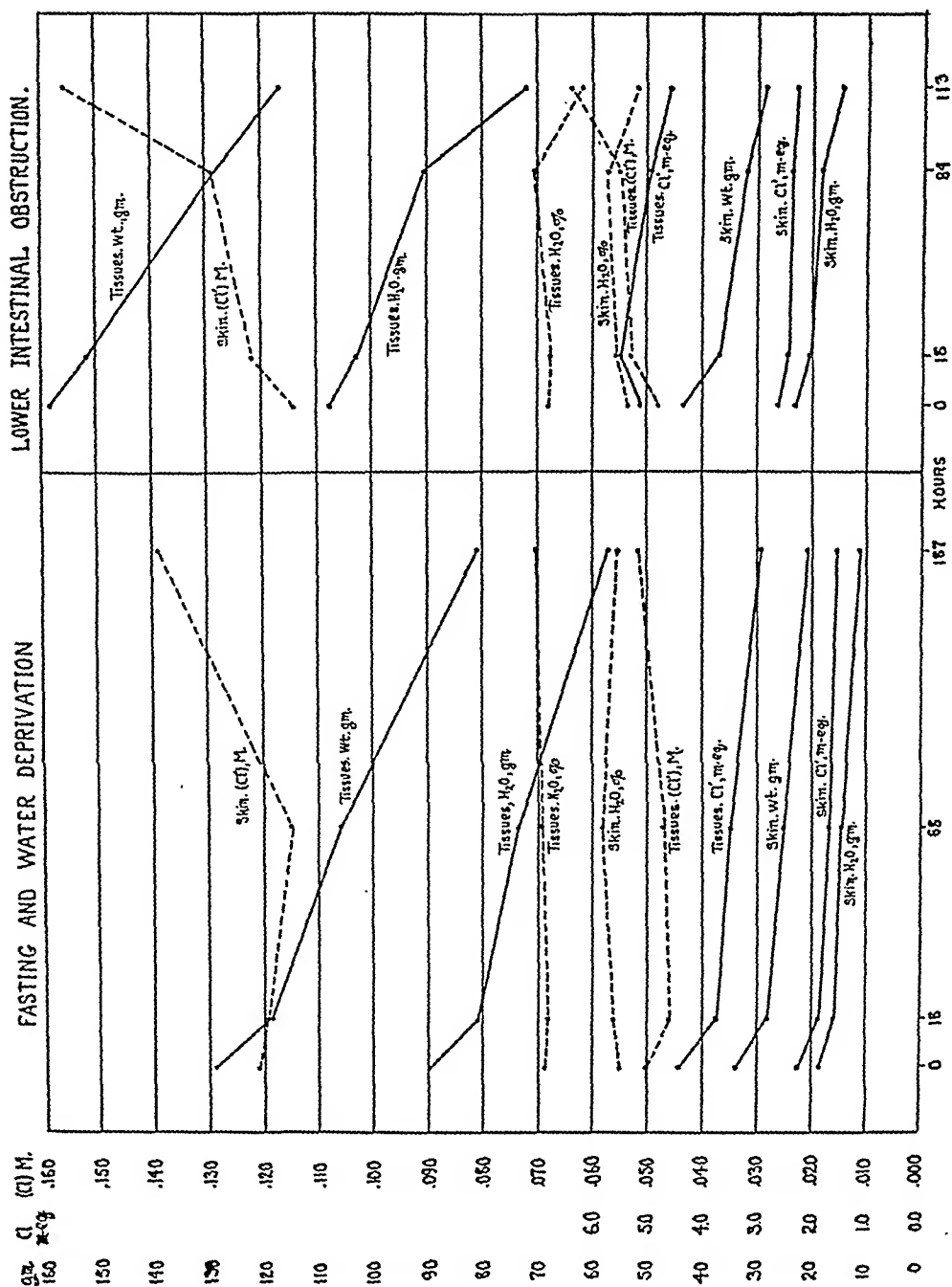


FIG. 2. Data representing effects of fasting with water deprivation, and of lower intestinal obstruction.

was placed at the lower end of the small intestine. Gastro-intestinal secretions can presumably be reabsorbed. Evidence that they are is provided by the rise in the concentration of chloride ion even during the initial period where, as may be seen in Fig. 1, pyloric obstruction produces a sharp fall. The survival period as indicated by a single animal was shorter than for fasting with water deprivation alone, 113 hours as compared with 187 hours. The two sets of data are, however, similar to a degree which permits the inference that lower intestinal obstruction causes little if any water removal from the body.

#### SUMMARY

The circumstances present in upper intestinal obstruction which may be expected to reduce the water content of the body are fasting with water deprivation and a continued loss of secretions into the stomach. According to the data obtained from the above described experiments with rats, loss of body water during the first third of the survival period following pyloric obstruction is more than half accounted for by fasting with water deprivation. This body water is accompanied by a parallel loss of solids and may be regarded as a waste product of the consumption of body fat, glycogen, and protoplasm. Its loss does not disturb the per cent water content of the body tissues. The water lost into the stomach is responsible for an actual excess of water reduction over consumption of solids. Except in the case of the skin and blood, this excess loss of water is extremely small and produces a reduction of the per cent water content of tissues which is so slight as to permit the surmise that the water loss here derives entirely from the interstitial fluid of the tissues and that no dehydration of tissue cells occurs. The data are, however, not directly informative on this point. The total loss of body water during 12 hours following pyloric obstruction was found to be 12.6 per cent of the water content of a control animal.

More than one-quarter (28.3 per cent) of the total body content of chloride ion was found to be lost and was entirely accounted for by the amount of chloride found in the gastric contents. Nearly half of the chloride loss derives from the skin.

Data are presented which demonstrate that lower intestinal obstruction causes slight, if any, depletion of the water content of the body.

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## STUDIES ON INFLAMMATION

### III. FIXATION OF A METAL IN INFLAMED AREAS\*

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(Received for publication, February 15, 1930)

In a previous communication (1) it was demonstrated that trypan blue injected intravenously rapidly enters an inflamed area and is fixed there so that the dye fails to reach the regional lymph nodes. Subsequent studies (2) revealed that the rate of fall of concentration of the dye in the capillaries as it diffuses into the extra-capillary spaces is more rapid in an inflamed than in a normal area. This was interpreted as being the result of increased capillary permeability with inflammation.

The earlier literature on the subject has been reviewed elsewhere (1). With the demonstration that the dissemination of bacteria was retarded by an inflammatory reaction (3, 4, 5) and with the subsequent studies on the fixation of dyes in inflamed areas, it became of interest to observe the behavior of other substances both when injected directly into an inflamed area and when injected into the blood stream. In some unpublished results it was found that carbon particles in the form of India ink failed to reach the tributary lymph nodes when injected directly either into a subcutaneous area of inflammation or when injected into an inflamed peritoneal cavity. When, however, the ink was injected intravenously no definite evidence of carbon deposit in the inflamed area could be obtained.

At the suggestion of Dr. Eugene L. Opie studies were then undertaken to determine whether a metal injected intravenously would accumulate rapidly in an inflamed area and be fixed there in a manner similar to trypan blue.

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*The Fixation of Iron by an Inflammatory Reaction in the Peritoneal Cavity*

The observations were made with the use of iron, largely because this metal could readily be detected when in sufficient amount in the tissues by placing them in an acidified solution of potassium ferrocyanide (Prussian blue reaction).

TABLE I  
*Retention of Iron in Inflamed Peritoneal Cavity*

Experiment	Interval between injection of irritant and that of iron*	Total duration of inflammation**	Prussian blue reaction in the retrosternal lymph nodes after injection of iron into the inflamed peritoneal cavity	Prussian blue reaction in the retrosternal lymph nodes after injection of iron into the normal peritoneal cavity
	hrs. : mins.	hrs. : mins.		
1	0 : 00	5 : 30	+	+
2	1 : 20	4 : 45	++	+++
3	3 : 05	5 : 45	0	+++
4	18 : 00	23 : 40	trace	++
5	21 : 30	28 : 00	0	+++
6	22 : 20	28 : 00	0	++
7	22 : 20	29 : 00	faint trace	+++
8	22 : 40	23 : 40	0	+++
9	23 : 15	28 : 00	0	++
10	23 : 15	28 : 45	faint trace	+
11	23 : 45	29 : 30	0	++
12	23 : 50	29 : 00	0	+
13	24 : 30	47 : 30	0	+++

0 indicates no Prussian blue reaction; + a pale blue reaction; ++ a conspicuous blue reaction; +++ a deep blue reaction.

\* In all except one experiment dialyzed iron was used. In Experiment 8 ferric chloride was used.

\*\* In all except one experiment aleuronat was used as the inflammatory irritant. In Experiment 4 a suspension of *Staphylococcus aureus* was used as the inflammatory irritant.

Opie (5) recently showed that acute inflammation of the peritoneal cavity caused by aleuronat retards the rush of injected hemolytic streptococci from the peritoneal cavity into the circulating blood and after 24 hours completely prevents it. Subsequently it was found (1) that trypan blue injected into the normal peritoneal cavity rapidly

appears in the lymph of the retrosternal lymphatics and stains deeply the retrosternal lymphatic nodes. Trypan blue, on the other hand, injected into an inflamed peritoneal cavity is fixed *in situ* and fails to reach the retrosternal lymphatic nodes.

5 cc. of dialyzed iron\* were injected into the peritoneal cavity of normal rabbits and also into that of rabbits with acute inflammation caused, with one exception, by a previous injection of 5 per cent aleuronat and 3 per cent starch in 0.5 per cent saline. The thorax was opened at various intervals after the injection of the metal and the retrosternal region dipped in 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid. Aleuronat *per se* gives a Prussian blue reaction but the evidence that none of the irritant appeared in the retrosternal lymphatic nodes is clear since in the animals with inflamed peritoneal cavity the retrosternal lymph nodes showed no Prussian blue reaction. The results are shown in Table I.

It is seen that when iron was injected immediately after the inflammatory irritant there was no retention of it (Experiment 1). When, however, the inflammatory reaction had been in progress for as long as 3 hours the metal was fixed and failed to reach the retrosternal lymphatic nodes. The injection of the metal into the normal peritoneal cavity was followed on the other hand by its appearance in the retrosternal lymphatic nodes. *Staphylococcus aureus* was used as the inflammatory irritant in one experiment (No. 4) with similar results, *i.e.*, fixation of the metal *in situ*. Ferric chloride (No. 8) behaved exactly as did dialyzed iron. In the inflamed peritoneal cavity where retention of the metal took place, large clumps and masses of brown deposits were often seen on the omentum and on the peritoneal surface of the diaphragm.

Having established qualitatively that colloidal iron and, in one instance, that ferric chloride are retained *in situ* by the inflammatory reaction, it became of interest to determine quantitatively the amount of fixation of the metal.

Quantitative determinations of iron in tissue were performed according to the method of Kennedy (6) with only slight modifications. 5 cc. of ferric chloride (0.25 per cent solution in saline) were injected into the peritoneal cavity of a normal rabbit as control and also into the inflamed cavity of an experimental animal. 1 to 2½ hours later the animal was killed and the retrosternal lymph

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\* This product is colloidal ferric hydroxide with 5 per cent ferric oxide and ferric chloride.



nodes carefully removed, weighed, and placed in a 100 cc. Kjeldahl flask. 2 to 3 cc. of concentrated sulfuric acid and about 1 cc. of 60 per cent solution of perchloric acid were added. The mixture was heated over a low flame for about 10 minutes until complete digestion took place and the solution appeared almost colorless. While the mixture was still hot, 0.5 cc. of 30 per cent hydrogen peroxide was added and the mixture was cooled and diluted to 50 cc. 10 cc. of this solution were pipetted into a 50 cc. stoppered cylinder to which were added 10 cc. of amylic alcohol and 5 cc. of 20 per cent sodium thiocyanate. The mixture was immediately shaken and the iron was extracted by the amylic alcohol layer. This layer was pipetted off into a colorimeter cup and compared with a standard treated in exactly the same manner.

TABLE II

*Amount of Iron in Retrosternal Lymph Nodes after Its Injection into Inflamed and Normal Peritoneal Cavity\**

Experiment	Interval between injection of irritant and that of iron	Total duration of inflammation	Amount* of iron in nodes after its injection into inflamed peritoneal cavity	Amount of iron in nodes after its injection into normal peritoneal cavity	Per cent of difference in iron content
	<i>hrs. : mins.</i>	<i>hrs. : mins.</i>			
1	17 : 30	18 : 30	165.7	222.7	25.6
2	22 : 30	25 : 00	195.7	762.5	74.3
3	22 : 30	25 : 00	38.5	180.95	78.7
4	24 : 30	26 : 00	86.0	127.0	48.0
Average of per cent difference in iron content.....					56.7

\* Figures are expressed in milligrams of iron per 100 gm. of tissue.

The results in Table II show that fixation of iron by an inflammatory process varies a good deal in different animals. These variations may partly be due to the fact that the determinations of iron content were performed by the "wet ashing" method, and probable differences in the amount of fluid in lymph nodes draining an inflamed and normal peritoneal cavity were therefore not taken into consideration in the final determinations. The average amount of iron in the retrosternal lymphatic nodes of normal animals is 56.7 per cent higher than in the nodes of animals with inflamed peritoneal cavity.

Three determinations were also performed to determine the iron content of retrosternal lymph nodes in animals receiving no iron. Two normal animals showed 35.6 and 44.8 mg. of iron per 100 gm. of

TABLE III  
*Accumulation of Iron at the Site of Cutaneous Inflammation*

Experiment	Duration of inflammation	Prussian blue reaction in inflamed skin areas of animals that received ferric chloride	Prussian blue reaction in inflamed skin areas of animals that receive no iron
	<i>hrs. : mins.</i>		
1	1 : 45	trace + + faint trace	0
2	4 : 30	++ ++ + +	0 0 0 0
3	6 : 15	+++ ++	0 0
4	6 : 30	++ +	0 0
5	8 : 00	trace to +	0
6	11 : 00	++ ++ ++ ++	0 0 0
7	23 : 30	+ ++ ++ +	0 0 faint trace
8	48 : 00		0 0
9	70 : 00		++ ++
10	168 : 00		+++ +++

0 indicates no Prussian blue reaction; + a pale blue reaction; ++ a conspicuous blue reaction; +++ a deep blue reaction.

tissue. One determination was made on the lymph nodes of an animal that had received no iron but had an inflamed peritoneal cavity. In this animal the value for iron content was 68.6 mg. per 100 gm. of tissue. These results with non-injected animals show that lymph nodes normally contain an appreciable quantity of iron, which is evidently bound up in a form that does not give the Prussian blue reaction. The Prussian blue reaction is less sensitive than the thiocyanate test for the detection of iron. Furthermore hemoglobin does not respond to the ordinary chemical test for iron. This probably accounts for the iron content of normal lymph nodes giving a negative Prussian blue reaction.

The above results show that like trypan blue, iron is fixed *in situ* by the inflamed cavity and much less of it reaches the retrosternal lymphatic nodes than under ordinary circumstances.

*The Accumulation of Iron Injected into the Circulating Blood in  
Inflamed Areas*

It having been found that iron was fixed *in situ* when injected directly into an inflamed area, studies were undertaken to determine whether, as in the case of trypan blue, the metal would accumulate in an inflamed area when introduced into the circulating blood stream.

25 mg. of ferric chloride in saline were injected intravenously. The areas of inflammation were caused by several injections of 0.1 to 0.2 cc. of a saline suspension of *Staphylococcus aureus* into the dermis of the abdomen of rabbits. The accumulation of iron was studied after the inflammatory reaction had been under way for various lengths of time. Inasmuch as it was found that an inflamed area *per se* of 70 hours duration or more gives a positive Prussian blue reaction owing doubtless to pigment from red blood corpuscles escaped by diapedesis or capillary hemorrhage, studies of iron accumulation in inflammation when ferric chloride was injected intravenously were not carried beyond 24 hours. The results are shown in Table III.

It is clear that the metallic salt accumulates in an inflamed area when the inflammatory process has lasted only 1 hour and 45 minutes. On the other hand the inflamed areas in non-injected animals give no qualitative test for iron within the first 11 hours of inflammation at least. The difference in the two sets of animals is conspicuous.

It is interesting to note that an old inflammatory lesion in non-

injected animals of at least 70 hours duration shows the presence of iron by the qualitative test. This is probably due to the degradation in the late stages of inflammation of hemoglobin from red corpuscles phagocytosed by tissue macrophages. Histological sections of such areas reveal within large mononuclear cells hemosiderin which gives the Prussian blue reaction. A similar observation in animals was recently made by Polson (7) while studying the fate of colloidal iron administered intravenously. However, this author does not refer it to the administration of iron. He was dealing with an inflammatory

TABLE IV

*Amount\* of Iron in Inflamed Areas of Animals That Received No Iron and of Animals Injected with Ferric Chloride*

Experiment	Injected animals		Non-injected animals	
	Normal skin areas	Inflamed skin areas	Normal skin areas	Inflamed skin areas
1	9.3	13.0	8.4	9.2
2	12.5	15.7	8.4	9.4
3	7.7	15.5	5.6	9.6
4	16.6	29.6	10.9	9.0
5	6.9	10.6	7.9	11.2
6	9.7	12.7	9.2	9.9
Average. . . . .	10.4	16.2	8.4	9.7

\* Figures are expressed in milligrams of iron per 100 gm. of dry tissue.

focus of relatively long duration which showed a large amount of iron in spite of the fact that no metal was injected.

Quantitative studies were undertaken to determine the exact amount of accumulation of metal in inflamed areas when 50 mg. of ferric chloride ( $\text{Fe}_2\text{Cl}_6$ , 12  $\text{H}_2\text{O}$ ) in distilled water were injected intravenously.

The inflamed areas were obtained by the injection into the dermis of the abdomen of about 0.2 cc. of a suspension of *Staphylococcus aureus* in broth concentrated to one-tenth of its original volume. 6 to 7 hours later the animal was killed and the inflamed areas were carefully removed, the subcutaneous tissue adhering to them being discarded. Control areas of normal skin in the same animal were also removed from the abdomen. These areas were then placed in an electric dry oven for about 16 hours at 100°C. When thoroughly dried the tissues were

weighed and digested by the method described above with the exception that now larger quantities of sulfuric and perchloric acids were used, the values being 8 and 5 cc. respectively. By determining the iron content of tissues on the dry basis, variations in the final results due to differences in fluid content of inflamed areas were largely eliminated. Thus a more accurate method of comparison between iron content of normal skin and inflamed areas was obtained. It is evident that with diapedesis of red corpuscles there is probably always more iron in an inflamed area than in a normal tissue area. For this reason an additional series of control animals was studied for iron content exactly as above with the exception that none of these rabbits received any intravenous injection of ferric chloride. The results obtained for iron content in inflamed areas in both groups of animals are shown in Table IV.

From this table it is seen that the average iron content in inflamed areas of animals injected with ferric chloride is 16.2 mg. per 100 gm. of dry tissue as compared with 9.7 mg. in inflamed areas of animals that received no metal. Consequently the amount of iron in inflamed areas is increased 67 per cent by injection of 50 mg. of ferric chloride in the blood stream. A comparison of the iron content of normal skin areas in injected and non-injected animals, 10.4 and 8.4 mg. respectively, shows that the accumulation in normal tissues resulting from the injection of the metal is only 23.8 per cent. Hence the accumulation of iron following intravenous injection of ferric chloride is about three times greater in inflamed than in normal skin areas. The iron recovered from inflamed areas of an animal not injected with this metal is contained in hemoglobin which does not give the Prussian blue reaction. This accounts for the negative reaction when the qualitative test is applied in these animals to inflamed areas of relatively short duration.

These experiments suggest that fixation of a metal or dye in an inflamed area may have clinical application. It is conceivable that dyes, iron containing substances, and various other materials by their accumulation at the site of inflammation may alter the character or course of the inflammatory reaction.

#### CONCLUSIONS

Colloidal iron or ferric chloride injected into the inflamed peritoneal cavity is fixed in the cavity and fails to reach the retrosternal lymphatic nodes, whereas, in the absence of inflammation, iron accumulates

in these nodes and becomes demonstrable by the Prussian blue reaction.

Quantitative studies show that after intraperitoneal injection of ferric chloride the retrosternal lymphatic nodes of animals with normal peritoneal cavity contain approximately 56 per cent more iron than do the nodes of animals with inflamed peritoneal cavity.

Ferric chloride injected into the circulating blood enters an inflamed area in the skin and the inflamed tissue gives the Prussian blue reaction. Quantitative determinations show that the amount of iron in inflamed areas is much greater than that found in inflamed areas of animals that have received no iron.

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# ACCUMULATION OF ANTIBODIES IN THE CENTRAL NERVOUS SYSTEM

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The purpose of the present paper is to describe the accumulation of antibodies in the central nervous system of rabbits. Information on this subject may throw light upon several important problems: (1) the penetration of protein (globulin) from the blood into the spinal fluid, brain and cord, for antibodies cannot be separated from globulins experimentally, (2) the relationship between the cerebrospinal fluid and the tissue fluid of the brain and spinal cord and (3) the prevention and treatment of certain diseases of the central nervous system by the injection of immune serums.

The present work is a continuation of a series of studies (1) on the antibody content of the serum and organs of rabbits, which have yielded information as to the distribution of agglutinins in the serum, organs and lymph. Agglutinins against typhoid bacilli were selected as antibodies because they can be obtained in high concentration and can be measured easily and with relative accuracy. The rabbit was employed because the titers of the immune serums of rabbits are very much higher than in other small laboratory animals.\* The experiments were carried out on actively and passively immunized animals. The active immunization consisted of a series of injections of killed typhoid bacilli into an ear vein. The passively immunized rabbits received one injection of immune serum into the blood stream. The antibody content of the organs was measured by extracting the organs (after grinding with sand) with salt solution and titrating the extracts. For the titration of agglutinins a method was used that is more sensitive than the usual routine method of agglutination. This method consists in centrifugalizing the tubes containing the serum or extract-dilutions and bacteria and reading the results while the tubes are being gently shaken and the sedimented bacteria resus-

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\* The dog—an animal extensively used in investigations on the central nervous system—does not furnish potent serum. Dogs could have been injected in the passive immunization experiments but by injecting immune serum obtained from rabbits into other rabbits the introduction of foreign protein is avoided.



pended. This technic was first used by Gathgens (2) in 1906 and has been employed by a number of investigators (3) since that time. For further technical details see previous publications of the present series.

The main results of the work, as already reported, can be summarized as follows: When serum containing antibodies is injected into an ear vein of the normal rabbit, antibodies accumulate in all the organs studied: the liver, spleen, kidney, lung, skin, muscle and uterus (smooth muscle). The rate of accumulation varies in the different organs. The final concentration is reached in the liver, spleen, lung and kidney in less than 10 minutes after the injection of immune serum, but in the uterus and skin only after several hours have elapsed. The antibodies penetrate most slowly into the skin. When the final amount of antibodies is accumulated there is a constant numerical relationship between the antibody content of the blood and organs. On the average, the highest dilution of extract prepared from 1 gm. of liver, spleen, kidney, lung or skin that agglutinated typhoid bacilli was one-tenth of the highest dilution of 1 cc. of serum that agglutinated typhoid bacilli.

Since there are less antibodies in the organs than in similar amounts of blood the question naturally arises whether the antibodies recovered from the organs are due to the blood present in them. That they are not derived mainly from the blood of the organs but from the extravascular part of the tissue is evidenced by the following observations. (1) Lymph obtained by cannulating the lymph ducts of the liver, leg, neck and thoracic duct contains antibodies in higher concentration than the organ extracts. (2) Perfusion does not reduce the antibody content of the skin and uterus. (3) More antibodies can be recovered in the perfusate from the living animal than were present in the blood before perfusion, showing that during perfusion antibodies penetrate from the organs into the blood vessels, an observation recently confirmed by Schwarzmann (4).

The equilibrium between the antibody content of the blood and of the organs can be reached from either the blood or the organs, for an identical relationship will establish itself between the antibody content of the serum and organs when the immune serum is injected either into the blood stream or into the skin.

#### EXPERIMENTAL

The experiments to be reported here were performed with both actively and passively immunized rabbits for these reasons. Passive immunization offers an opportunity to establish the rate of accumulation of antibodies in the organs and cerebrospinal fluid by examination of the rabbits at different intervals of time after the injection of immune serum. In actively immunized rabbits the titers of the blood and organ extracts are higher and therefore the observations are more striking. The technic of immunization was the same as in the previous work. The immune serum used was fresh, and was obtained and kept under sterile conditions.

Before describing the experiments, it must be emphasized again that the agglutination tests were made with the aid of centrifugalization, a method that is more sensitive than the routine method of agglutination. Without this technic, the results described in the present study cannot be duplicated.

*I. Antibody-Content of the Brain, Spinal Cord and Cerebrospinal Fluid (from Cisterna Magna) of Actively Immunized Rabbits*

The technic of these experiments differed from that of the earlier experiments in that urethane was not used for anesthesia because it is said that it promotes the

TABLE I

*Agglutinin Titers of the Serum, Spinal Fluid, Brain and Spinal Cord of Actively Immunized Rabbits*

Number of rabbit	Serum	Spinal fluid	Brain	Cord
1	1:150,000	1:640	1:2,500	1:1,300
2	1:150,000	1:420	1:600	1:384
3	1:128,000	1:160	—	—
4	1:102,000	1:512	1:576	1:1,200
5	1:102,000	1:256	1:1,500	1:362
6	1:102,000	1:512	—	—
7	1:102,000	1:512	—	—
8	1:102,000	1:512	1:583	1:290
9	1:50,000	1:128	—	—
10	1:32,000	1:80	1:200	1:104
11	1:32,000	—	1:192	1:60
12	1:32,000	1:40	—	—
13	1:26,000	1:48	—	—
14	1:20,000	1:52	—	—
15	1:13,000	1:26	—	—
16	1:3,200	1:5	—	—

passage of substances into the spinal fluid. The animals were narcotized with the minimum amount of ether necessary. Some of the rabbits were bled to death from the left carotid artery; some from the femoral arteries and the descending aorta; the site of bleeding did not influence the results. After the rabbits were bled to death cerebrospinal fluid was removed from the cisterna magna by means of a tuberculin syringe and skin-test needle (gauge 22). After some practice there was no difficulty in obtaining fluid free of blood. The samples of cerebrospinal fluid—in most cases 0.4 cc.—were centrifugalized and the sediment examined under a

microscope. Samples containing more than one red blood cell in 10 microscopic fields (seen with high dry lens) were rejected. From the brain and spinal cord the meninges were removed very carefully and the ventricles of the brain and central canal of the cord were then opened and carefully rinsed with salt solution. To ascertain whether any cerebrospinal or other fluid containing agglutinins was left on the surface of the organs, the last washing fluid was examined for agglutinins. Some of the washing fluid caused a trace of agglutination, but dilutions 1 in 2, 1

TABLE II

*Agglutinin Titers\* of the Spinal Fluid and of Extracts of the Brain and Spinal Cord of Actively Immunized Rabbits*

Number of rabbit	Spinal fluid	Brain extract	Spinal cord extract
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.43	1.70	0.87
2	0.28	0.40	0.26
3	0.12	—	—
4	0.50	0.56	1.17
5	0.25	1.47	0.35
6	0.50	—	—
7	0.50	—	—
8	0.50	0.57	0.28
9	0.26	—	—
10	0.25	0.62	0.32
11	—	0.60	0.18
12	0.12	—	—
13	0.18	—	—
14	0.26	—	—
15	0.20	—	—
16	0.20	—	—
Average.....	0.33	0.82	0.49

\* The titers are expressed as percentages of the titers of the serum.

in 4 did not agglutinate typhoid bacilli. The washed organs were dried by pressing them lightly between filter papers, weighed, and ground with sea sand alone and with saline. The extracts were centrifugalized at high speed, the sediment discarded, and the supernatant fluid centrifugalized again until it became clear. In recording the results of titration, titer 1:100 means that when 1 gm. of brain was extracted with 9 cc. saline, the extract, diluted ten times, clumped typhoid bacilli and a dilution twice higher—1:200—did not agglutinate typhoid bacilli.

Tables I and II show the following.

1. The cerebrospinal fluid of all the rabbits immunized with killed

typhoid bacteria contained antibodies. The titer of the cerebrospinal fluid varied with the titer of the serum, the ratio of the titer of the serum to the titer of the cerebrospinal fluid being, on an average, 300:1, or 0.33 per cent. The variation in this numerical relationship exceeded in one case only the limit of accuracy of the method. One should bear in mind, in this connection, that in the agglutination test the serum, cerebrospinal fluid and organ extract are diluted by halves (1 in 100, 1:200).

2. The brain and the spinal cord of all the actively immunized animals contained agglutinins. The titer of the extracts of the brain and cord varied with the titer of the serums, but the numerical relationship between the titers of the serums and organ extracts was not so consistent as the numerical relationship between the titers of the serums and the cerebrospinal fluid. The ratio of the titers of the serums to the titers of the extracts of the brain and of the spinal cord ranged from 100:0.4 to 100:1.7 and from 100:0.18 to 100:1.17 respectively. The average agglutinin titer of brain extract was 0.82 per cent (of the titer of the serum) and that of the spinal-cord extract 0.49 per cent (of the titer of the serum). Therefore extracts prepared from 1 gm. of brain or spinal cord were more potent than the dilution of a similar amount of cerebrospinal fluid.

Summarizing the results of the observations on actively immunized rabbits, the following average numerical relationship was found between the agglutinin titers of the serum, spinal fluid, brain and spinal-cord extracts:

Blood serum.....	100.	per cent
Cerebrospinal fluid.....	0.33	per cent
Brain extract.....	0.82	per cent
Spinal-cord extract.....	0.49	per cent

## *II. Antibody-Content of the Central Nervous System of Passively Immunized Rabbits*

In studies of the distribution of antibodies in the blood and organs of passively immunized animals it is of great advantage to employ immune serums of high titers; therefore only very potent serums were used for passive immunization. The majority of the animals were injected with immune serums of which the titer was 1:64,000. The titer of one of the immune serums was as high as 1:200,000.

Fig.

The titer of the serums and the amount injected are given in Table III. The serum was introduced slowly into an ear vein through a skin-test needle (gauge 22), the injection lasting from 3 to 4 minutes. Since it is generally believed that substances in solution injected into a peripheral vein are evenly distributed in the blood stream within 3 minutes, six rabbits were bled to death as early as 15 minutes after the immune serum had been injected. The results of the titrations of the serums, cerebrospinal fluids and organ extracts are tabulated in Tables III and IV.

TABLE III

*Agglutinin Titers of the Serum, Spinal Fluid, Extracts of Brain and Spinal Cord of Passively Immunized Rabbits*

Number of rabbit	Serum injected		Time between injection of serum and examination	Serum	Spinal fluid	Extract of brain	Extract of spinal cord
	Amount	Titer					
1	10 cc.	1:128,000	15 minutes	1:25,000	0	1:80	1:80
2	10 cc.	1:128,000	15 minutes	1:25,000	0	1:50	1:50
3	25 cc.	1:64,000	15 minutes	1:16,000	1:5	1:96	1:29
4	25 cc.	1:64,000	15 minutes	1:24,000	less than 1:2.5	1:240	1:84
5	15 cc.	1:64,000	15 minutes	1:12,000	1:6	1:264	1:180
6	15 cc.	1:64,000	15 minute	1:12,000	less than 1:12	—	—
7	10 cc.	1:128,000	2 hours	1:18,000	1:1.8	1:68	1:18
8	10 cc.	1:128,000	2 hours	1:25,000	—	1:120	1:50
9	10 cc.	1:80,000	2 hours	1:10,000	1:6	1:120	1:80
10	25 cc.	1:64,000	3 hours	1:12,000	1:3.6	—	—
11	20 cc.	1:64,000	3 hours	1:12,000	1:32	—	—
12	15 cc.	1:200,000	4 hours	1:32,000	1:19	1:294	1:126
13	20 cc.	1:64,000	18 hours	1:6,400	1:11	1:29	1:10
14	25 cc.	1:64,000	18 hours	1:3,200	1:8	1:13	1:5
15	20 cc.	1:32,000	20 hours	1:3,200	1:3.2	—	—
16	20 cc.	1:64,000	20 hours	1:4,800	1:18	—	—
17	20 cc.	1:64,000	20 hours	1:3,200	1:10	—	—
18	20 cc.	1:64,000	24 hours	1:3,200	1:11.5	—	—
19	20 cc.	1:200,000	24 hours	1:40,000	1:160	—	—

The tables show that in four rabbits, which were bled beginning 10 to 15 minutes after the injection of immune serum and ending 10 minutes later, the undiluted cerebrospinal fluid failed to agglutinate typhoid bacilli. However, in two other rabbits a very small amount of agglutinins penetrated into the cerebrospinal fluid within that time. The penetration of the antibodies into the cerebrospinal fluid (obtained from the cisterna magna) proceeded at a slow rate, for the high-

est titers in the cerebrospinal fluid were found only after 15 hours had elapsed following the injection of immune serum. In these animals the titer of the cerebrospinal fluid ranged between 0.10 to 0.40 per cent of the titer of the serum; that is, it was about as high as in actively immunized rabbits.

TABLE IV

*Agglutinin Titers\* of the Cerebrospinal Fluid and of Extracts of the Brain and Spinal Cord of Rabbits Following Intravenous Injection of Immune Serum*

Number of rabbit	Time between injection of serum and examination	Titer of cerebrospinal fluid	Titer of brain extract	Titer of extracts of spinal cord
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	15 minutes	0	0.32	0.32
2	15 minutes	0	0.20	0.20
3	15 minutes	0.03	0.60	0.18
4	15 minutes	less than 0.01	1.00	0.35
5	15 minutes	0.05	2.2	1.5
6	15 minutes	less than 0.1	—	—
7	2 hours	0.01	0.38	0.10
8	2 hours	—	0.48	0.20
9	2 hours	0.06	1.20	0.80
10	3 hours	0.03	—	—
11	3 hours	0.27	—	—
12	4 hours	0.06	0.92	0.43
13	18 hours	0.17	0.45	0.15
14	18 hours	0.25	0.40	0.14
15	20 hours	0.10	—	—
16	20 hours	0.37	—	—
17	20 hours	0.31	—	—
18	24 hours	0.36	—	—
19	24 hours	0.40	—	—

\* The titers are expressed as percentages of the titers of the serums.

Tables III and IV show that just as in actively immunized rabbits, the extracts of the brain and cord of passively immunized rabbits contained agglutinins. The numerical relationship of the titers of the serums and extracts of brain and cord are very similar to those found in the actively immunized animals, the titer of brain extract being on an average 0.70 per cent and that of the cord 0.71 per cent of the titers of the serum. The variation from the average was more marked than in the actively immunized rabbits.

It was expected that the accumulation of antibodies in the central nervous system would proceed at a slow rate. This was found true of the penetration of agglutinins into the cerebrospinal fluid. However it had not been expected that the titers of the organ extracts of rabbits examined 15 minutes after the injection of immune serum would be as high as those from rabbits that had been in contact with immune serum for 24 hours. This unexpected observation suggests either that the penetration of antibodies into the brain and spinal cord (tissue fluid of these organs) is as fast as into some organs, such as the spleen, liver and lung and faster than into the uterus and skin, or that the antibodies recovered were due mainly to the blood present

TABLE V

*Antibody Titers of Serums and of Extracts of the Perfused Brain of Actively Immunized Rabbits*

Number of rabbit	Titer of serum	Titer of brain extract	Titer* of brain
			<i>per cent</i>
1	25,000	270	1.0
2	25,000	180	0.7
3	12,800	770	0.6
4	250,000	1280	0.5
5	128,000	640	0.5

\* Expressed as percentages of the titers of the serums.

in the brain and spinal cord. To throw light on this question, I perfused the brain of rabbits of which the blood serum had a high titer in the agglutinin test.

### *III. Antibody-Content of the Cerebrospinal Fluid, Brain and Spinal Cord after Perfusion*

The perfusion experiments were performed as follows. An actively immunized rabbit was lightly narcotized with ether, and about 30 cc. of blood was obtained from the left femoral artery. Then 0.15 gm. of heparin was injected into an ear vein; 3 minutes later the rabbit was bled to death from the femoral arteries and the abdominal part of the descending aorta. Immediately after the bleeding was completed, the brachial arteries and descending part of the aorta were ligated, and Locke solution at 42°C. was introduced into the arch of the aorta with the purpose of perfusing the brain through the vertebral and internal carotid arteries. The

perfusion was usually continued for about 1 hour; about 500 cc. of perfusion fluid was used. The examination of cerebrospinal fluid, and of organ extract was carried out as described above.

Table V shows that perfusion did not diminish the antibody titers of the brain extract or the cerebrospinal fluid. However it was felt that before drawing a conclusion from this observation as to the presence of the antibodies in the extra-vascular brain tissue, it was desirable to obtain evidence of the adequacy of the perfusion experiments. To this end I compared histological sections of the brains of the exsanguinated rabbits with those prepared from rabbits whose brain was perfused. The comparison showed that in the unperfused brains the majority of the blood vessels contained red blood cells whereas in the perfused brains the blood vessels were distended and red blood cells were absent from the majority of them.

Final evidence for the view that the antibodies recovered from the brain by extraction are derived from the extra-vascular part of the tissue would be the demonstration of antibodies in the lymph flowing from the brain. However, no lymph duct draining the brain is known; therefore no direct evidence can be furnished at the present time for this view.

#### DISCUSSION

The antibody content of the cerebrospinal fluid of normal and diseased human beings and lower animals has been the subject of extensive clinical and experimental investigations. It has been very generally accepted that antibodies do not penetrate from the blood into the cerebrospinal fluid unless the meninges are inflamed. A survey of the literature, however, shows that several authors have reported the presence of antibodies in the cerebrospinal fluid without inflammation.

Hektoen and Carlson (5) found opsonins but no haemagglutinins in the cerebrospinal fluid of actively or passively immunized dogs. Becht and Greer (6) could not demonstrate agglutinins in the cerebrospinal fluid of rabbits immunized with typhoid vaccine. Kafka (7) found traces of hemolysins and bacterial agglutinins in the cerebrospinal fluid of immunized dogs. Starkenstein and Zitterbart (8) reported that only undiluted cerebrospinal fluid of dogs agglutinated typhoid bacilli, although the titer of their serum was as high as 1:10,000.



There are two reports in the literature on the relative titers of tetanus antitoxin in the serum and cerebrospinal fluid. Ransom (9), working in von Behring's laboratory, compared the antitoxin titers of the serum and of the cerebrospinal fluid of one very highly immunized horse. He found that the ratio of the antitoxin titer of the serum of this animal to that of the cerebrospinal fluid was 100:0.4. Lemaire and Debre (10), who studied the effect of morphine upon the permeability of the meninges, reported that in dogs injected with tetanus antitoxin the ratio of the titer of the serum to that of the cerebrospinal fluid was 100:0.2.

The experiments performed in the present study show clearly that antibodies are present in the cerebrospinal fluid of actively or passively immunized animals without inflammation of the meninges. The possible objection that the antibodies demonstrated were due to inflammation or contamination of the cerebrospinal fluid with blood as a result of faulty technic can be met as follows. (1) The specimens of cerebrospinal fluid did not contain red blood cells at all or only in a negligible number. (2) There was a constant numerical relationship of the titers of serums to the titers of cerebrospinal fluids. (3) In passively immunized rabbits the antibody titer of the cerebrospinal fluid increased during the first 15 hours following the injection of immune serum, although the titer of the blood decreased during this time.

It is pertinent to inquire whether the central nervous system of the rabbits used in these experiments was free of pathological changes. McCartney (11) at the suggestion of Flexner, examined the brains of a large number of apparently healthy rabbits and found histological evidence of meningo-encephalitic lesions in more than 50 per cent. His observation is in conformity with those made in other laboratories (Bull (12), Oliver (13)).

The brains of rabbits employed in the present work were not examined histologically, but it is reasonable to assume that the lesions found by McCartney occur in the brains of our stock rabbits.

McCartney, Bull and Oliver did not study the spinal fluid of their rabbits and therefore it is not known whether the lesions found by them in the brain are associated with a large number of leucocytes or with other signs of inflammation in the cerebrospinal fluid. Although the spinal fluid of the rabbits employed in the present work was free from an abnormal number of leucocytes, the possibility cannot be excluded that meningo-encephalitic lesions so prevalent in apparently normal

rabbits did not influence the accumulation of antibodies in the central nervous system in some of them. However antibodies were found in the spinal fluid, brain and spinal cord in all of the actively and all of the passively immunized rabbits 2 hours after passive immunization. It hardly seems possible that the accumulation of antibodies in the central nervous system of all the rabbits could have been due solely to the presence of meningo-encephalitis.

The reasons for the general belief that antibodies do not penetrate from the blood into the cerebrospinal fluid without the inflammation of the meninges are probably the following. (1) The antibody titer of the cerebrospinal fluid is relatively low, as would naturally follow from the circumstance that the antibody titers of the serums of animals examined in the course of the reported studies were not high. (2) A large number of studies dealt with normal hemolysins, whose titer in the serum is very low.

Amoss and Eberson (14) reported that "agglutinins were not found in the spinal fluid of normal monkeys which had received antimeningococcic serum intravenously." This observation can be readily explained by the experiments on rabbits reported here, which show a ratio of 300:1 between the titers of the serum and of the spinal fluid; whereas the serum of the monkey in the experiment of Amoss and Eberson contained only 100 units of agglutinins per cubic centimeter, and the amount of agglutinins present in the spinal fluid was, therefore, too small to be detected. Flexner, Clark and Amoss (15) found that "it is unusual for the neutralizing principles to be contained in the cerebrospinal fluid during convalescence from epidemic poliomyelitis," although neutralizing principles are present in the blood. It is, however, not probable that the serums of the convalescents contained the neutralizing principles or antibodies in quantities that could be demonstrated in three-hundred-fold dilution.

Although, as my experiments show, antibodies penetrate from the blood into the spinal fluid of rabbits even without inflammation, the fact remains that the antibody content of the spinal fluid is very small. The results of numerous clinical and experimental observations have shown the effectiveness of sterile inflammation and of injecting immune serum into the spinal fluid in raising the antibody content of the spinal fluid (Flexner). In experiments to be reported later this finding has been reobtained in rabbits.

Since antibodies are found in the globulin fraction of the serum, behave in many respects like globulins and may be expected to follow the distribution of globulins, it is interesting to compare the ratio of the antibody titers of the serum and the cerebrospinal fluid on

the one hand with the ratio of the globulin of the serum and that of the cerebrospinal fluid on the other hand. Mestezrat (16) stated that the globulin content of the cerebrospinal fluid is about 0.019 gm. per 100 cc., and it is said that 100 cc. of serum contains on an average 2.5 gm. of globulin. These data were obtained from the serum and cerebrospinal fluid of man, but it is possible that the globulin content of the serum and cerebrospinal fluid in the rabbit is at least of similar magnitude if not almost equal to that found in human beings. The ratio of globulin content of the serum to that of the cerebrospinal fluid,

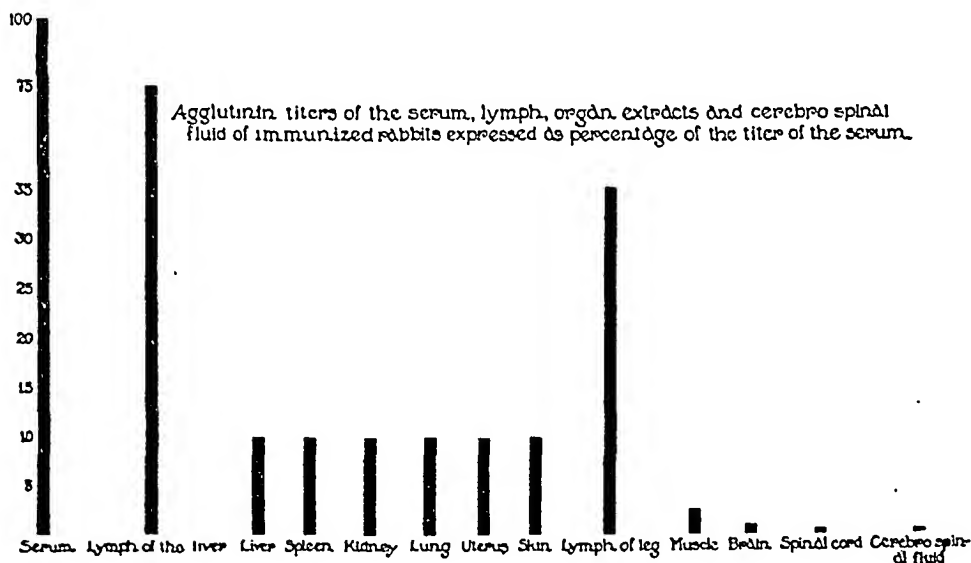


CHART 1

100:0.76, nearly equals that of the respective antibody titers, 100:0.30. Considering the accuracy of the technic of agglutination tests a closer agreement can hardly be expected. Therefore it can be said there is some parallelism between the antibody titers of the serum and cerebrospinal fluid on the one hand and the globulin content of these fluids on the other hand.

These observations have an obvious bearing on the serum therapy of the central nervous system. They show that antibodies do accumulate in the tissue of the brain, spinal cord and cerebrospinal fluid even if the immune serum is not injected into the central nervous sys-

tem; furthermore, that the antibody content of the central nervous system can be estimated by titration of the blood.

It is important to ascertain whether antibodies accumulate in the tissues of the central nervous system at a faster rate when the immune serum is injected into the spinal fluid instead of the peripheral blood stream. This question is being investigated and will be the subject of a subsequent publication.\*

In the previous studies it was found that the agglutinin titers of the extracts of the spleen, liver, lung, kidney, uterus and skin are about the same and on the average ten times lower than that of the serum (extract of 1 gm. of organ compared with dilution of 1 cc. of serum). The titer of extracts of the muscles of the leg is lower than those of the other organs examined, varying from 1 to 5 per cent expressed as percentage of the titer of the serum. In contrast to these organs the brain and cord yield extracts that contain antibodies in very low titer, less than 1 per cent of the titer of the serum. (See Chart 1.)

#### CONCLUSIONS

1. Antibodies can be extracted from the brain and spinal cord of rabbits actively or passively immunized with typhoid bacilli.

2. The titers of the antibodies in the extracts of brain and cord depend upon the titer of the blood serum. In actively immunized rabbits the following numerical relationships exist between the titers of the serum and of these organ extracts: The ratio of the titer of the serum is to the titers of extract of brain and of the spinal cord about as 100 is to 0.8; the titer of the serum is to the titer of the cerebrospinal fluid as 100 is to 0.3. In passively immunized rabbits the titer of the serum is to the titer of brain and spinal-cord extract as 100 is to 0.7.

3. The antibodies recovered from the brain are not due to the presence of blood in it for perfusion of the brain does not reduce its antibody content appreciably.

4. Antibodies penetrate into the spinal fluid from the blood even in the absence of inflammation of the meninges. When the penetration is completed the following numerical relationship exists between the titer of the serum and that of the cerebrospinal fluid: 100 to 0.25.

\* More general discussion of the literature will be published in a subsequent paper.

5. The penetration into the cerebrospinal fluid of antibodies injected intravenously proceeds at a slow rate, being completed only several hours after the immune serum has been injected. The penetration of antibodies into the tissue of the brain occurs at a very rapid rate. It is completed within 15 minutes.

6. It is very unlikely that when the immune serum is injected intravenously the antibodies reach the brain tissue by way of the cerebrospinal fluid, for (1) the antibody titer of the cerebrospinal fluid is lower than that of the brain extract, and (2) antibodies penetrate faster into the tissue of the brain than into the cerebrospinal fluid.

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# CULTIVATION AND CLASSIFICATION OF "BACTEROIDES," "SYMBIONTS," OR "RICKETTSIAE" OF *BLATTELLA* *GERMANICA*

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The writer<sup>1</sup> recently reported the isolation and cultivation of the intracellular microorganism within the large American roach, *Periplaneta americana*, and found that it was a bacterium belonging to the genus *Corynebacterium* (the diphtheroids). Three types could be distinguished on the basis of certain morphological and serological characters. The species was named *Corynebacterium periplanetae* nov. sp. variety *americana*.

It seemed important to determine whether other closely affiliated insects harbor similar intracellular parasites, and whether the parasite can be adapted to artificial media by using the technical procedures already described. The German roach or "croton bug" (*Blattella germanica*) was selected for investigation.

Sections through the eggs, embryos, and body of *Blattella* show the existence of bacteriocytes with an arrangement and morphology similar to those of *Periplaneta*. The cytoplasm of these cells is packed with bacteria-like microorganisms which may be liberated by triturating fresh tissue in physiological salt solution. As in the case of *Periplaneta*, they are non-motile, non-encapsulated bacilli which are not acid-fast but Gram-positive. The microorganisms stain well by Giemsa's method and are seen to be extremely pleomorphic, with equal and unequal division or budding. The bacilli are straight or crescent-shaped, banded or bipolar, and often terminate in a thickening or club. Short, oval, and coccoidal forms are also common, and appear to represent buds produced from the longer rods. Spores have never

<sup>1</sup> Glaser, R. W., *J. Exp. Med.*, 1930, 51, 59.

been observed. Chains of three units are sometimes seen. The individual units of the bacillary forms have a mean length of approximately  $2.5\mu$  with a modal length of  $3.2\mu$ . The breadth varies from  $0.8$  to  $1.6\mu$ . The diameter of the coccoidal forms was fairly constant, approximately  $0.8\mu$ . The above enumerated facts undoubtedly place the species in the genus *Corynebacterium*.

The procedures for isolating *Corynebacterium periplanetae* free from contaminants and for cultivating the species on dextrose horse blood agar by means of the "spotting technique" have been fully described. The same methods were adopted for the *Blattella* parasite which was isolated and cultivated from the bacteriocytes of six adults and from four embryos removed from egg capsules. Altogether 86 spots were made from these and 10 cultures obtained. Approximately 12 per cent of the attempts succeeded.

The cultivated form corresponds closely to the intracellular parasite. All cultures are non-motile, non-encapsulating, non-sporulating bacilli not acid-fast but Gram-positive. Giemsa's stain shows the same characters previously mentioned, namely, pleomorphism, banding, flexing, with club, oval, and coccoidal forms. In addition cultures on dextrose horse blood agar show many small cocci, diplococci, and *Rickettsia*-like stages. The purity of these cultures can hardly be questioned because they have been repeatedly plated and always yield, in 120 hours, one type of clear, round colony of pinpoint size.

Serological tests with the ten *Blattella* isolations demonstrated the presence of two strains. These strains and their intracellular forms were compared with the *Periplaneta* intracellular parasite and its three cultural types. A morphological differentiation between the intracellular forms was not possible.

Table I represents a series obtained by making 25 measurements for each character. The preparations were fixed in equal parts of absolute alcohol and ether for 15 minutes and stained according to Giemsa's method.

The sizes of the cultural forms reasonably correspond to the sizes of their intracellular counterparts. Slight differences in the mean length of the bacilli occur, but the modal length of the intracellular forms, whether from ova or from mature bacteriocytes, is fairly constant. The cultures show some slight differences from the intracellular forms

and from one another. A perusal of both the mean and modal length of all bacillary forms shows that Type II from *Periplaneta* is the longest and Type III from the same host the shortest. Other differences between the three *Periplaneta* types were pointed out in the earlier work.

Strains I and II from *Blattella* are indistinguishable from one another on the basis of size, but may be segregated from the *Periplaneta* cultural types by the large number of minute coccoidal forms which appear in cultures of the former.

TABLE I

Source of parasites	Length range	Mean length	Modal length	Breadth range	Diameter range of coccoidal forms
	$\mu$	$\mu$	$\mu$	$\mu$	$\mu$
<i>Periplaneta</i> bacteriocytes.....	2.0-5.0	3.0	3.2	0.5-1.0	0.5-1.0
<i>Blattella</i> " .....	1.6-4.0	2.5	3.2	0.8-1.6	0.8
<i>Periplaneta</i> ova.....	0.8-4.8	1.9	3.2	0.5-1.0	0.5-1.0
<i>Blattella</i> " .....	0.8-4.8	2.4	3.2	0.8-1.2	0.8
<i>Periplaneta</i> cultures:					
Type I.....	0.5-3.2	1.9	3.2	0.4-0.6	0.5-1.0
"    II.....	1.6-4.8	3.4	4.0	0.5-1.0	0.5-1.0
"    III.....	1.6-3.2	2.1	2.4	0.4-0.6	0.3-0.8
<i>Blattella</i> cultures:					
Strain I.....	0.8-4.8	2.6	3.2	0.4-0.8	minute-0.8
"    II.....	1.6-4.0	2.8	3.2	0.8-1.2	minute-1.2

The *Blattella* strains cannot be differentiated from those of *Periplaneta* culturally nor on their fermentation reactions to carbohydrates as Table II shows. None of the forms produce gas. They ferment glucose, sucrose, and maltose, but not lactose nor mannite.

Table III represents some agglutination tests with *Blattella* Strains I and II against normal rabbit serum and against rabbit serum immunized against the three *Periplaneta* types.

These tests were performed at the same time as those described in the previous work, so that a comparison is possible. Such a comparison shows that the *Blattella* strains are distinct from those of *Periplaneta*. Serologically Strain II from *Blattella* resembles Type III from *Peri-*



TABLE II

Carbohydrates.....	Glucose	Lactose	Sucrose	Maltose	Mannite
pH of medium.....	7.3	7.3	7.3	7.3	7.3
<i>Periplancta</i> :					
Type I.....	5.2	7.3	5.2	5.3	7.4
" II.....	5.2	7.3	5.0	5.3	7.4
" III.....	5.4	7.2	5.6	5.3	7.3
<i>Blattella</i> :					
Strain I.....	5.4	7.3	5.5	5.4	7.3
" II.....	5.1	7.2	5.5	5.3	7.3

TABLE III

## Agglutination Tests

Normal rabbit serum and *Periplancta*, Types I, II, and III immune rabbit sera against the *Blattella* diphtheroid, Strains I and II. Suspensions of microorganisms in 0.20 per cent NaCl solution. Standardization on Gates turbidity scale to 2.0.

Serum immune to	Titer of immunizing culture	Antigen used	Serum dilutions									NaCl
			1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	
<i>Periplaneta</i> :		<i>Blattella</i> :										
Type I...	1/160	Strain I	-	-	-	-	-	-	-	-	-	-
		" II	++	++	+	+	-	-	-	-	-	-
" II...	Clumps spon- tane- ously	Strain I	-	-	-	-	-	-	-	-	-	-
		" II	-	-	-	-	-	-	-	-	-	-
" III...	1/10,240	Strain I	-	-	-	-	-	-	-	-	-	-
		" II	C	C	C	+++	++	++	+	+	±	-
Normal serum		Strain I	-	-	-	-	-	-	-	-	-	-
		" II	-	-	-	-	-	-	-	-	-	-

*plancta* more closely than the other forms. Distinctions also exist between the two *Blattella* forms.

For the species of intracellular diphtheroid found within *Blattella germanica*, the writer would like to propose the name *Corynebacterium blattellae* nov. sp.

## SUMMARY AND CONCLUSIONS

In *Blattella germanica*, the German roach or "croton bug," bacteriocytes are found in all individuals of both sexes. These bacteriocytes are scattered throughout the fat tissue and their cytoplasm is filled with microorganisms. Evidence is presented to show that the intracellular parasites are diphtheroidal bacilli. These diphtheroids are transmitted from one generation to another through the ova.

By using a technic previously described, the intracellular parasites were isolated and cultivated from the adult bacteriocytes and from embryos. Two diphtheroidal strains were cultivated with approximately equal frequency. These two strains resemble one another closely enough to be considered a single species but show certain minor differences. The sizes, general morphology, and tinctorial reactions of the two cultures correspond to the intracellular parasites of *Blattella germanica*. They may be distinguished from the three types of *Corynebacterium periplanetae* variety *americana*, previously described. For the species here discussed the name *Corynebacterium blattellae* nov. sp. is proposed.



# A CONTRIBUTION TO THE EPIDEMIOLOGY OF SPECIFIC INFECTIOUS CYSTITIS AND PYELONEPHRITIS OF COWS

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PLATES 28 AND 29

(Received for publication, March 7, 1930)

In previous papers (1,2) we called attention to the presence of a specific disease of the urinary tract of cows which had been known in Europe for many years but had not heretofore been recognized in this country. From our material we concluded that the bladder is first involved with secondary involvement of the ureters and kidneys. A bacterium of the diphtheroid type similar to that obtained by several workers in Europe was readily cultivated from the inflamed portions of the urinary tract. In our experiments when small quantities of pure cultures were introduced into the bladder by way of the urethra, a slowly spreading cystitis resulted but the inflammatory process failed to ascend the ureters. Certain bacteriological and other clinical studies failed to confirm the generally accepted view that the urinary infection was associated with genital disease and we were unable to obtain the specific organism from sources other than the urinary tract. Bacteriological examination of the urine of the bulls of two herds in which the disease was prevalent failed to reveal the specific diphtheroid.

It has been difficult to explain the mode of spread within the herd, although certain experiments in which cows were stabled in close contact with others known to be infected resulted in transmission. It was the practice to curry and brush the cows twice a day and probably the infectious material was repeatedly introduced into the vagina by means of brushes. In some other instances the source of infection was not evident. The propagation of the organism seems to lie within

the urinary tract of infected cows. Under usual herd conditions transmission thus becomes a difficult matter, since septic material is not usually brushed into the vagina. Some natural means of propagation must necessarily exist and our problem concerned itself with this question.

Dr. Theobald Smith (3), in his examination of the urine of calves, frequently encountered diphtheroids in large numbers, and he has suggested that they might be of significance. It was with the idea of correlating these organisms with those from bladder infections of adult cows that the following studies were made.

### *Method*

In the main the calves were drawn from one herd from which the bulk of our original material had been obtained. A group of the calves were brought to the Institute and used for other experiments, and at autopsy the genito-urinary tract was turned over to us for examination. These were considerably older than the calves from which we obtained material at the abattoir. The calves slaughtered at the Institute varied from 3 weeks to 2 months of age, while those killed at the abattoir were from a few days to 2 weeks old. In a few instances we obtained material from heifers 3 to 4 months old, but most of the material came from males.

The genito-urinary tract was removed in one piece and the whole tract seared. The urine was withdrawn from the bladder and the tract incised from the bladder to the sheath. Cultures were prepared from the bladder mucosa, the upper, middle and lower portions of the urethra, and the sheath, by scraping the mucosa vigorously with a curette and streaking the surface of blood agar plates with the scrapings. The plate cultures were incubated for 48 hours and suggestive colonies picked for inoculation into other media. When material from female calves was examined, scrapings from the bladder, urethra and vagina were made. The urine obtained from the bladder was centrifuged and plate cultures prepared from the sediment. The urinary tracts of 34 calves were examined.

The cultures which conformed in general to the diphtheroidal type were propagated, and, if regarded of sufficient interest, studied on differential media. In this way it was found that several distinct cultural groups existed among the strains. This has been brought out in Table I.

Table I indicates that the diphtheroids fall into 5 cultural groups. It has been shown that the bacterium associated with specific cystitis and pyelonephritis ferments dextrose and fails to ferment the other substances. It produces enough alkali in litmus milk to turn the litmus a deep purple and finally the medium becomes sufficiently alka-

TABLE I  
*The Cultural Characters of Diptheroids Isolated from the Urinary Tract of Calves*

Group	No. of strains	No. of calves from which isolated	Final hydrogen ion concentration in					Effect on milk	Effect on gelatin	Chromogenesis on potato
			Dextrose	Lactose	Saccharose	Maltose	Mannitol			
I	25	12	4.9-5.3	7.4-8.0	7.4-8.0	7.4-8.0	7.4-8.0	Becomes alkaline; casein dissolved	Not liquefied	Varies from white to faint cream to orange
II	44	18	5.4-5.8	7.6-8.0	6.6-5.0	6.6-5.2	7.6-8.0	"	"	Grayish yellow to orange
III	11	8	5.6	8.0	8.0	6.0	8.0	"	"	Orange
IV	8	3	5.2-5.4	8.0	7.6-8.0	6.8	7.6-8.0	"	"	Cream to orange
V	40	16	7.4-8.0	7.4-8.0	7.4-8.0	7.4-8.0	7.4-8.0	Remains unchanged or becomes alkaline; casein dissolved	"	Yellow to orange

line to dissolve the casein. From Table I it is evident that we succeeded in isolating a large number of strains of proper morphology which possessed the biochemic properties of the strains isolated from the disease. The table also indicates that other types of diphtheroids occur frequently in the urinary tract. Some of them when freshly isolated are readily distinguishable morphologically from those of the first group. Their biochemic features lead to a ready differentiation. It should be stated that all types were non-motile and stained intensely by the Gram method.

The distribution of the various cultural types throughout the genito-urinary tract is of interest and is shown in Table II.

TABLE II  
*The Distribution of the Various Types in the Genito-Urinary Tract*

Group	Isolations from				
	Sheath	Urethra	Bladder	Urine	Vagina
I	16	5	0	3	1
II	7	28	8	1	0
III	1	4	5	1	0
IV	3	4	1	0	0
V	10	11	10	6	3

Cultural Type I, which we regarded as of considerable significance, seems to be confined largely to the sheath. Its entire absence from the bladder is noteworthy although certain strains were recovered from the lower portion of the urethra. On the other hand, the second cultural group seems to live largely in the urethra and bladder, since 36 of 44 strains were isolated from these regions. Group V occurs throughout the urinary tract and has been isolated from the vagina. The other two groups contain such small numbers that their distribution seems to have little meaning.

Since the first group possessed all the morphological and cultural characters manifested by the organism associated with the specific infection in the adults, further correlations were manifestly desirable. With this in view a number of strains were chosen for further study. Rabbits were immunized with cultures isolated from the kidneys of

TABLE III  
*The Agglutination Affinities of the Group I Strains Obtained from the Urinary Tract of Calves*

The Agglutination Affinities of the Group 1 Ovarian Secretions															
Culture	Dilutions of serum of rabbit immunized with culture from spontaneous case							Dilutions of serum of rabbit immunized with culture from Calf 1474 sheath							Control
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:5	1:10	1:20	1:40	1:80	1:160	1:320	
Cow 1132 (left kidney)	C*	C	C	++	++	++	++	C	C	C	++	++	++	++	-
Calf 1474 (sheath)....	C	C	++	++	++	++	++	C	C	C	C	++	++	++	-
" 1494 " .....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	-
" 1539 (urethra)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	B
Cow R 418 (urine)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	-
Calf 1533 (sheath)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	-
" 305 (urethra)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	B
" 1540 (urine)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	+
" 306 (urethra)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	+
" 1480 (urine)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	+
" 303 (sheath)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	-
" 307 " .....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+
" 1480 " .....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	-
" A " .....	++	++	++	++	++	++	++	++	++	++	++	++	++	++	B
Cow D. M. (urine)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	B
Calf 1531 (sheath)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	-
" 1522 (urine)....	C	C	++	++	++	++	++	C	C	C	++	++	++	++	+

\* C = complete. The quantity of agglutination has been recorded as +, a definite but slight clumping, to ++, a heavy clumping without clearing. B = spontaneous precipitation in the form of a button at the bottom of the tube.



spontaneous cases and with certain typical strains from calves. When sera with sufficient agglutinin were obtained all strains were tested. That these possessed agglutination affinities similar to those of the organisms isolated from the kidneys of adults in spontaneous infections is shown in Table III.

On the whole it can be said that the strains resembling the etiological type behave in a manner similar to those isolated from spontaneous cases in cows. The serum from a typical calf strain is capable of agglutinating those from the cows and *vice versa*.

TABLE IV  
*The Effect of Reciprocal Absorption on Agglutination*

Culture	Serum dilutions of culture Cow 1132 serum absorbed with culture Calf 1474						Serum dilutions of culture Calf 1474 serum absorbed with culture Cow 1132						Control
	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320	
Cow 1132 (kidney)...	±	—	—	—	—	—	+	±	—	—	—	—	—
Calf 1474 (sheath)...	—	—	—	—	—	—	±	—	—	—	—	—	—
“ 1494 “ ....	+	+	—	—	—	—	—	—	—	—	—	—	—
“ 1539 (urethra)...	+	—	—	—	—	—	—	—	—	—	—	—	—
Cow 418 (urine).....	+	±	—	—	—	—	+	—	—	—	—	—	—
Calf 1533 (sheath)...	+	±	±	±	±	±	+	+	±	±	±	±	±
“ 1540 (urine).....	±	±	±	±	±	±	±	±	±	±	±	±	±
“ 306 (urethra)...	+	±	±	±	±	±	++	±	±	±	±	±	±
“ 1480 (urine).....	+	—	—	—	—	—	±	—	—	—	—	—	—
“ 303 (sheath)...	±	±	±	±	±	±	+	±	±	±	±	±	±
“ 1480 “ ....	—	—	—	—	—	—	—	—	—	—	—	—	—
“ A “ ....	—	—	—	—	—	—	—	—	—	—	—	—	—
Cow D. M. (urine)...	±	—	—	—	—	—	+	+	±	±	±	—	—
Calf 1531 (sheath)...	—	—	—	—	—	—	—	—	—	—	—	—	—
“ 1522 (urine).....	±	±	—	—	—	—	±	±	—	—	—	—	—

In the course of the correlation it seemed advisable to test the antigenic specificity further. With this in mind, the serum produced by immunization with the strain obtained from a cow's kidney was absorbed with the culture obtained from the sheath of Calf 1474 and the other serum was absorbed with the culture isolated from the kidney. The absorbed sera were then tested with the various strains as shown in Table IV.

The results of the tests of the sera after absorption indicate that

a typical strain obtained from the sheath of a healthy calf is capable of absorbing practically all the agglutinin from the antiserum specific for the etiological strains. The same held true when the serum produced by immunization with one of the calf strains was absorbed with a culture from an adult infection. The cultural and immunological findings support the view that the organisms are similar.

The calves from which cultures were obtained were apparently normal. This brought up the question of pathogenicity. It is known that freshly isolated cultures when injected into rabbits or guinea pigs in considerable quantities fail to produce lesions. Our previous experiments indicated that small quantities of culture from either bladder or kidney infections when introduced into the cow's bladder by means of a catheter gave rise to a slowly progressive persistent cystitis. The most important test of identity seemed to us to be that of the pathogenic properties of the strains. Four cows were available for the tests and these were injected with various cultures. The details are given in the following protocols. The urine from all was examined twice at intervals before the injections were made. Cultures failed to show significant diphtheroids and the urine was free from albumin and other abnormal products.

On April 22, 1929, 4 cows were inoculated intra-urethrally with 3 cc. of 48 hour broth cultures, as follows: Cow 1517, culture from the urine in spontaneous infection of Cow R 418. Cow 1520, culture from the lower urethra of Calf 1539. Cow 1530, culture from the sheath of Calf 1539. Cow 1559, culture from the sheath of Calf 1480.

One week later the urine of all was examined. The urinary sediment of all contained diphtheroids similar in morphology and cultural characters to those inoculated. Six days after the first examination specimens were again examined. The urine from Cows 1517, 1520, and 1530 showed little abnormal; that from Cow 1559 was turbid and gave a strong reaction for albumin. One week later the urine from all 4 cows was reexamined with the same results. The diphtheroids had disappeared from the urinary sediment of 1517, 1520, and 1530. The specimen from 1559 contained blood, gave a strong albumin reaction, and was rich in red cells, clots and diphtheroids. It was at this time that the first urethral hemorrhage was observed. Urination became more frequent and considerable straining following micturation, often accompanied by the passage of bright red blood. A sample of urine obtained 3 months after the inoculation revealed blood, mucus, and large numbers of diphtheroids.

Cow 1559 was slaughtered at an abattoir 6 months and 21 days after inoculation.

The whole genito-urinary tract was brought to the laboratory for examination. The bladder was contracted, its walls thickened, and the vessels of the subserosae dilated. The whole mucosa was congested, swollen and wrinkled, and scattered over its surface there were many irregularly rounded raised hemorrhages. On the floor of the bladder were a number of irregular, slightly raised, flattened, adherent patches which were removed with difficulty, exposing a raw, weeping surface. The mucosa between and about the outlets of the ureters appeared as a wrinkled, greatly swollen, reddened, spongy mass. Both ureters were dilated and the walls markedly thickened. The right was almost half again as large as the left (Fig. 1). The mucosa of both appeared edematous and was streaked here and there with longitudinal red lines. Portions of two lobes of the right kidney contained irregularly rounded, grayish white, homogeneous areas which involved only the cortex. Several irregular, sharply circumscribed accumulations of yellowish green caseous material were present at the tips of the papillae (Fig. 1). The areas varied from 1 to 2 mm. in diameter to nodules as large as 1 cm. (Fig. 2). The caseous material shelled out easily revealing a glistening capsule. Films from the caseous areas revealed enormous numbers of diphtheroids. Portions of the mucosa of the pelvis were swollen and reddened (Fig. 1). The mucosa of the ureter where it left the kidney revealed a similar process (Fig. 1). In addition to these findings a cyst which protruded from the vaginal wall was noted. Diphtheroids were readily obtained from the urine at autopsy. Films from the pelvic abscesses showed diphtheroids in enormous numbers to the exclusion of other forms. Cultures developed only diphtheroids. The left kidney revealed no abnormalities.

Pieces of the bladder, ureter and right kidney fixed in Zenker's fluid revealed well marked changes. Microscopic examination of sections of the bladder showed a variety of lesions. The mucosa in one area was largely intact but the submucosa was greatly thickened, edema was pronounced, accumulations of round cells and hyperplasia of the connective tissue accompanied by capillary proliferation were remarkable. In other portions little of the mucosa remained except for a few streamers of degenerated cells. The exposed surface consisted of a richly cellular submucosa composed of proliferating connective tissue containing many vessels and heavily infiltrated with round cells. The left ureter presented little abnormal except edema of the submucosa and proliferation of the submucous connective tissue. The mucosa of the right ureter revealed degenerative changes and some exfoliation. The deeper portions of the mucosa were invaded by round cells. The submucosa was edematous and rich in capillaries as well as moderately infiltrated with round cells.

The left kidney presented no serious derangement. The section from the right revealed little abnormal in the cortex. The medulla presented no abnormality until close to the papillus, where some of the tubules revealed exfoliation of the mucosa. There was a moderate engorgement of the capillaries. Nearer the pelvis the desquamation was more marked and the vascular engorgement

more pronounced. About the tip of the papillus and extending a uniform depth into the kidney a well defined lesion was observed. On the whole the tubular outlines were well preserved but the mucosa had disappeared and the lumina were plugged with homogeneous material. Hemorrhages into the tubules were not infrequent. Under lower magnifications several deep blue staining, clear cut masses lying close to the papilla and having the outlines of large tubules were readily discerned (Fig. 3). These when sufficiently magnified proved to be tubules of which only the basement membrane remained, and the blue staining areas were composed of densely packed masses of diphtheroids (Fig. 4). On the whole the lesion impressed us as being relatively recent and was characterized by its sharp definition and its relative freedom from cellular reaction. The remarkable growth of the causative organism within the tubules with consequent interference with the neighboring circulation and the absorption of bacterial products might well account for its character.

The other 3 cows were killed 24, 39, and 53 days after inoculation. The bladders and kidneys failed to show abnormalities and the organisms could not be recovered from the urine.

It will be seen from the protocols that two of the three cows inoculated with cultures obtained from the lower urinary tract of calves developed only transient infections. The inoculation in the third instance (Cow 1559) produced cystitis, pyelitis and pyelonephritis. The cow injected intra-urethrally with culture from the kidney of a spontaneous infection developed only transient cystitis.

#### DISCUSSION

The findings indicate that the lower genito-urinary tract of male calves may harbor an organism similar in morphology, cultural characters and certain immunological properties to the organism encountered in this country and Europe in spontaneous infections of the bladder, ureter and pelvic portions of the kidneys of cows. The organism has been found in the sheath, urine, and lower portions of the urethra in 12 of 34 calves examined. By far the greatest number of strains were obtained from the sheath. The mucosa of the sheath and the retained urine harbor bacteria in enormous numbers and the diphtheroid seems to make up but a small proportion of the organisms present.

The findings are significant from an epidemiological standpoint. As we have pointed out, the difficulty in tracing infection from animal to animal within the herd has been great. It seems probable that many cases may really originate as infections during early life. The

organisms are able to maintain themselves on the mucous membranes of the lower urinary tract where little damage is done, but by a gradual, ascending growth they may finally reach the upper urethra and bladder. Where the tube is short as in the female the supposition is reasonable; and in this sex additional complications, such as pressure on the bladder, albuminuria, etc., may act to quicken the invasive process. Unfortunately, our material contained only a few female calves; but from one of these we obtained from the vagina in the region of the urethral orifice a culture indistinguishable culturally and immunologically from the pathogenic type. The source of the fairly frequent sheath infestation is not easily traced. In one instance a calf born of an infected mother was examined and the organism recovered from the sheath. The same organism was readily cultivated from the mother's urine and in all probability the sheath was directly inoculated during parturition. In five other instances the urine of the mothers of calves in which we had cultivated the diphtheroid from the sheath was examined with negative results.

The brief citation of a spontaneous infection in a 12 day old male calf serves as evidence that infection may begin in early life.

The genito-urinary tract was obtained immediately after slaughter. The kidneys presented no gross abnormalities. The bladder walls were greatly thickened and the mucosa swollen and edematous. Hemorrhages varying in size from minute points to considerable extravasations were scattered over the mucosa. The urine within the bladder was turbid and contained blood. The urinary sediment was rich in red cells, round cells, leucocytes, and diphtheroids. Typical diphtheroids were obtained in pure culture. Microscopic examination of fixed and stained material from the bladder revealed the usual type of cystitis encountered in adult infections of this kind.

Certain facts (2) indicate that the sheath of the adult male does not contain many organisms of the etiological type, since we failed to cultivate them from naturally passed urine in 11 instances, although organisms similar to those of Groups II and IV were readily grown.

From the experiment in which 3 cows were artificially infected with strains from calves and only 1 developed the disease, it might be suggested that certain of the calf strains are not pathogenic. It is true that all the cultures employed in the injection had been under artificial cultivation for several months and that that from the spontaneous

case was isolated 2 years before the inoculation. The strain which produced the infection in the inoculated cow had been isolated about 1 year before it was used. On the other hand, as a control procedure, a cow was inoculated with a culture obtained from the kidney of a severe case but this gave rise only to transient cystitis. From this it can be argued that the resistance of certain cows might explain our results.

We have produced cystitis in a fair proportion of inoculations, but the disease has thus far been confined to the bladder. In the instance of the cow which we regard as most susceptible (1559) culture introduced into the bladder produced the local process and later reached the pelvis of one kidney by way of the ureter. The experimental evidence indicates that under favorable conditions diphtheroids introduced into the bladder may produce cystitis, pyelitis and pyelonephritis.

#### SUMMARY

Bacteriological examination of the genito-urinary tract of calves originating in a herd in which infectious cystitis and pyelonephritis exists among the cows, revealed a variety of cultural types of diphtheroids. Of these types, one obtained from a considerable number of the calves resembled in morphology and cultural characters the organism cultivated from the actual cases of the disease. This group had agglutination affinities like those of the organism mentioned and was capable of absorbing agglutinin from antiserum specific for it. When three cows were inoculated intra-urethrally with cultures isolated from the sheaths of calves, two developed transient infections and the other a severe prolonged cystitis and pyelonephritis.

#### REFERENCES

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## EXPLANATION OF PLATES

## PLATE 28

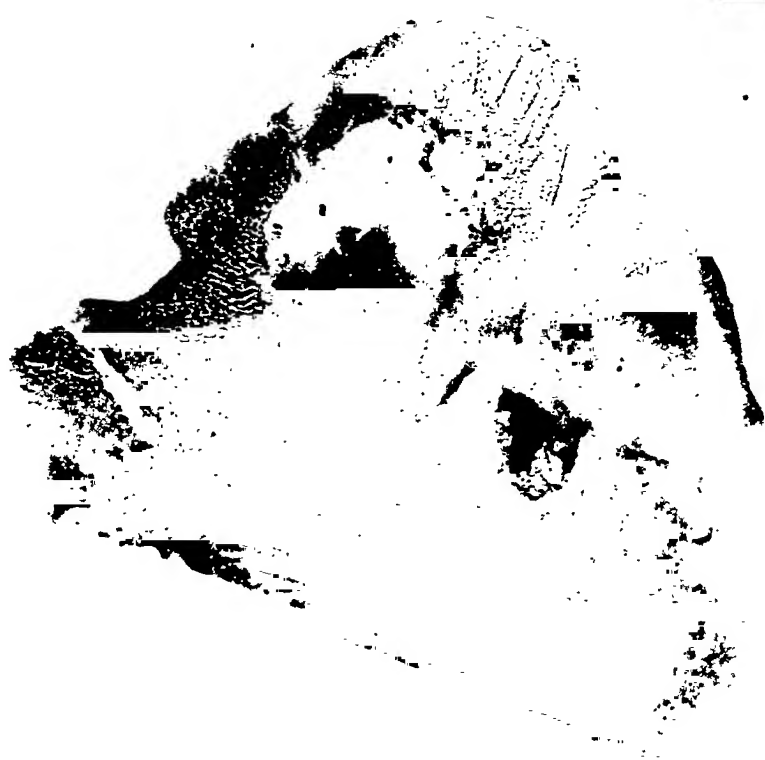
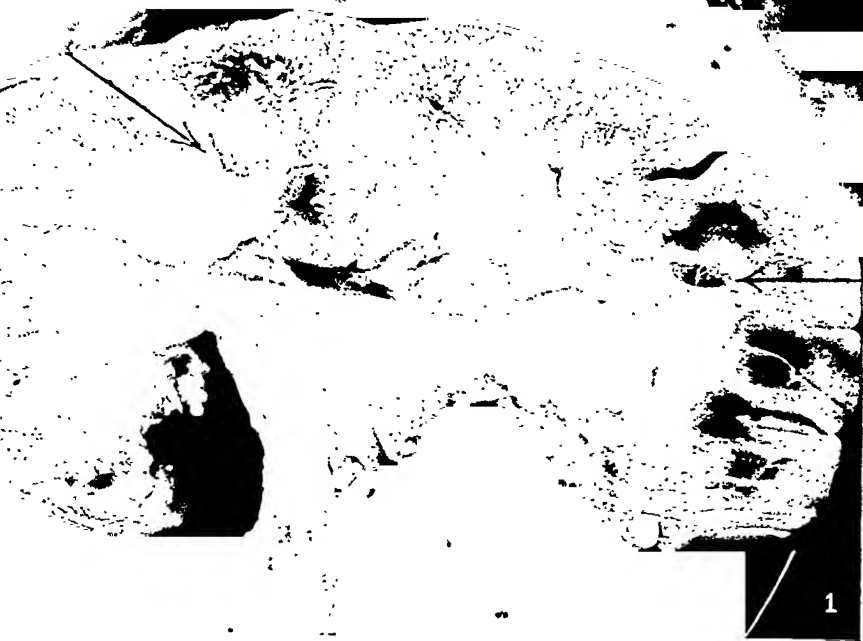
FIG. 1. The right kidney of Cow 1559 which had been inoculated intra-ureth-  
rally with culture obtained from the sheath of a normal calf. Note edema and  
congestion of the pelvic mucosa, the size and appearance of the ureter. The  
arrows indicate the location of small abscesses. About  $\frac{2}{3}$  actual size.

FIG. 2. The largest abscess encountered in another portion of the right kidney.  
X about  $1\frac{1}{4}$ .

## PLATE 29

FIG. 3. Section of the right kidney of Cow 1559 showing an early lesion deep  
within the medulla. Note the degenerative changes and the intensely black  
tubules due to deeply stained bacterial masses within them. Zenker's fixation;  
eosin and methylene blue stain. X90.

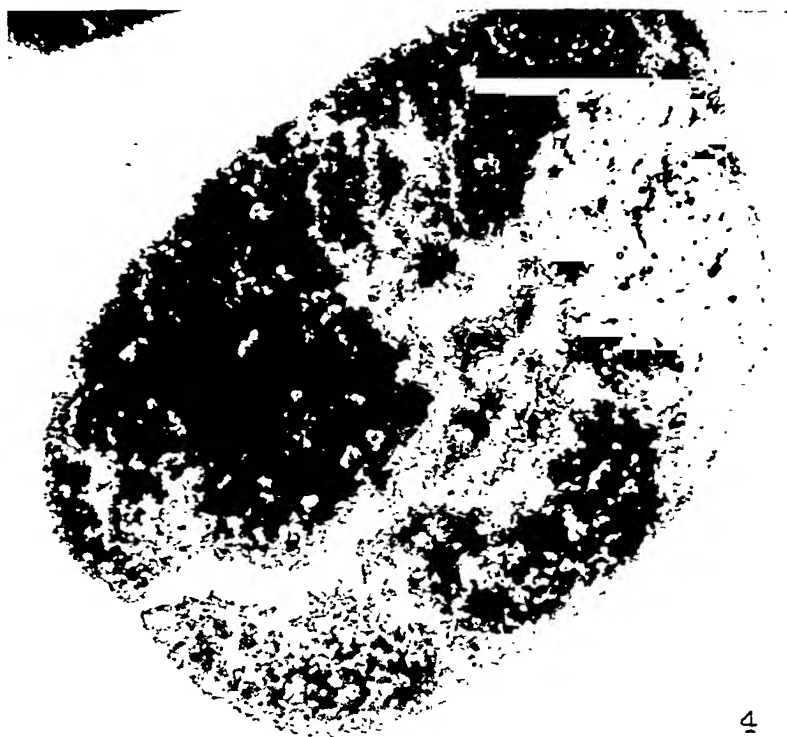
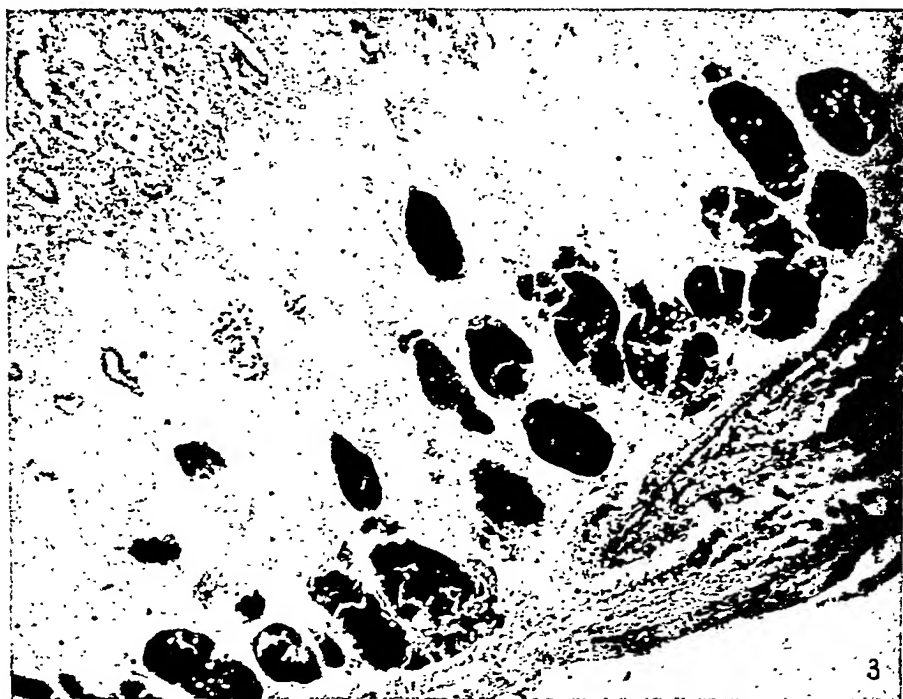
FIG. 4. A cast composed of tightly packed masses of the diphtheroid from a  
single tubule from the same preparation. X660.



(Jones and Little: Cystitis and pyelonephritis of cows)









# THE EFFECT OF CATHODE RAYS UPON CERTAIN BACTERIA

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PLATE 30

(Received for publication, March 26, 1930)

A study of the rate of killing of bacteria by cathode rays is a step towards finding out what changes these radiations and X-rays bring about in cells. The great interest to biology of X-rays, and probably also of cathode rays, lies not in the fact that they are lethal, but rather in their ability to produce permanent and inheritable changes in cell properties. Nevertheless, because they are deadly it is obvious that an understanding of the conditions under which cells are killed is a necessary preliminary to any thorough-going study of how they are changed. Bacteria have been used in the following experiments partly because they are, on the whole, the simplest cells to work with, partly because much has already been done on the lethal action of X-rays upon them, and partly because through their small size they provide the best introduction to the examination of the effects of these radiations upon the still smaller and imperfectly understood viruses.

## *Experimental Procedure*

In the experiments to be described in this paper, single bacteria spread upon an agar plate are bombarded with a known number of cathode rays and, after incubation, the number of survivors is determined through counts of the colonies of bacteria. From these data and various well known physical characteristics both of the rays and of the bacteria, a satisfactory picture can be gained of many details of the destruction of these cells.

The cathode rays were obtained from a Coolidge type electron tube<sup>1</sup> leased from the General Electric Company. This tube is essentially a large hot cathode X-ray tube with a hollow anode covered by thin metal foil. Many electrons proceeding from the cathode pass through this anode and foil and, emerging into the air, constitute the cathode ray beam. The velocity of these electrons and, in general, the transparency of matter to them increases with the voltage applied to the tube. For these experiments the voltage was approximately 155 K.V. The velocity of the emitted electrons is of the order of 0.8 of the velocity of light.

The absorption of an electron in inanimate matter is attended by the release of a large number of ions within a very small volume. A 150 K.V. electron will liberate about  $10^4$  ions within less than 0.001 mm.<sup>3</sup> It is natural and in accord with existing knowledge to associate the changes brought about in a bacterium by cathode rays with the absorption of electrons and the consequent shower of ions freed within it. Together with this ionic shower, X-rays are emitted as a consequence of electron absorption. The passage of cathode rays through air is likewise attended by the formation of much ozone and it might be argued that the observed destructive action on bacteria was due either to these X-rays or to ozone. We have ascertained by suitable experiments that this is not the case.

Several conditions must be fulfilled if a statistical analysis of the killing of bacteria by cathode rays is to have any physical significance. In the first place, the absorption of these rays is so great that the bacteria must be exposed upon the surface of an agar plate rather than held in a suspension if the dose they receive is to be satisfactorily measured. It is likewise necessary that they be spread upon this surface with sufficient uniformity so that colony counts in selected standard areas can be taken as measures of the numbers of irradiated bacteria. Not only must the bacteria be spread as single organisms, but their multiplication must be prevented until irradiation is completed. This is evident when it is realized that if several organisms are associated together in a clump, a single survivor will produce a countable colony. Many experimental procedures were tried with several types of bacteria before one was found which gave a satisfactory spread of single organisms. The highly motile *B. aertryke* gave excellent results with 200 to 300 organisms per square inch and almost as satisfactory data were provided by *B. coli* in somewhat greater dilution. Repeated trials, however, failed to produce as good spreads of single organisms of *Staphylococcus aureus*. This bacterium has frequently been used in

<sup>1</sup> Coolidge, W. D., *J. Franklin Inst.*, 1926, 202, 693.

studies of the action of X- and ultraviolet rays. Our experience makes it seem probable that single cell spreads were not ordinarily obtained in these experiments.

The bacteria were prepared for irradiation in the following way. Broth cultures, from standards provided by Dr. L. T. Webster of this Institute, were carried for at least 3 days through daily transplants before use. The final 20-hour old tube was diluted an hour before irradiation using either physiological salt or Locke's solution. This dilute suspension, so chosen as to give the desired number of bacteria per plate, was kept in an ice bath until actually used in order to prevent

TABLE I  
*Survival Ratios in Typical Experiments*

Time	Survival ratios				
	(1) <i>B. coli</i>	(2) <i>B. aerl.</i>	(3) <i>B. aerl.</i>	(4) <i>B. aerl.</i>	(5) <i>S. aureus</i>
4 sec.	0.698	0.562	—	—	0.853
5 "	.610	.417	0.699	0.671	.745
8 "	.435	.310	.602	.612	.566
12 "	.311	.258	—	—	.341
14 "	—	—	.481	.372	—
16 "	.204	.174	—	—	.248
20 "	.061	.151	.321	.264	.169
24 "	.050	.103	—	—	.168
28 "	.026	.055	—	—	.205
Electrons incident per cm. <sup>2</sup> /sec.	$1.65 \times 10^9$	—	$0.85 \times 10^9$	$0.96 \times 10^9$	$2.99 \times 10^9$

cell multiplication. In seeding, 1 cc. of this suspension was run evenly over the agar surface of a poured 10 cm. Petri dish. After vigorous shaking to remove the excess fluid, the plate was allowed to drain 10 minutes and its edges wiped free from adhering liquid. A known area was then immediately irradiated and another marked as standard. After incubation over night, counts were made of the colonies developing in each region. Approximately 100 plates were irradiated for each experiment. In some experiments the plates themselves were refrigerated before being inoculated. This proved to be unnecessary and had the disadvantage that the attendant condensation of moisture on the agar surface often destroyed the bacterial distribution.

The plate ready for irradiation was placed in the holder C of Fig. 1 and its agar surface pressed against the cutting edge of the tube A. This tube is divided

lengthwise into two hemicylinders by a brass and lead partition. The electrons stream through the lower of these halves from the tube window, D, and strike the surface of the agar pressed into the cutting edge. The upper half, sealed with lead to exclude the electrons, provides a standard adjacent to the irradiated surface and of equal area. Cutting through the agar with the tube A has the double advantage of defining sharply the irradiated area and of marking permanently both it and the standard. The tube end was sterilized between irradiations by wiping with alcohol. The time of irradiation was controlled by operating a heavy lead shutter shown at B. In counting the number of electrons emerging from the tube, the measuring device to be described later replaced the plate, other conditions being the same as during irradiation.

In each experiment involving about 100 plates eight different times of exposure were commonly used and 10 or more plates were irradiated for each of these times. The ratios of the colony counts made after incubation upon their irradiated and standard areas were averaged for each time of exposure to give the typical results of Table I.

### *Analysis*

These data can be analyzed with the help of the following considerations from elementary probability theory. If  $a'$  is the probability that an event will take place, by definition the probability of its not happening is  $b' = 1 - a'$ . Furthermore, if  $c'$  is the probability that some other event will occur, then the probability that  $a'$  and  $c'$  will happen together is  $a'c'$ . In the experiments of this paper many electrons are being shot at a few bacteria. If  $a$  is the probability that an electron will hit a bacterium and  $n$  is the number of electrons shot at it, the probability that one of these electrons will hit and that every other electron will miss it is

$$a(1-a)^{n-1}$$

Since any one of the  $n$  electrons may hit, the probability of striking a bombarded bacterium only once is

$$P_1 = na(1-a)^{n-1}$$

Similarly, the probability of hitting a bacterium by each of two selected electrons is  $a^2$  and the probability of doing it only twice and by these two out of the  $n$  electrons is

$$a^2(1-a)^{n-2}$$

If there are  ${}_nC_2$  ways of combining two out of  $n$  electrons, the probability that a bacterium is hit twice and only twice is

$$P_2 = {}_nC_2 a^2 (1-a)^{n-2} = \frac{n(n-1)}{2} a^2 (1-a)^{n-2}$$

In the same way, the probability that a bacterium will be struck  $r$  out of  $n$  possible times is

$$P_r = {}^nC_r a^r (1-a)^{n-r} = \frac{n(n-1)(n-2)\dots(n-r+1)}{r!} a^r (1-a)^{n-r}$$

The number of electrons striking the irradiated surface per second is of the order of  $10^{10}$ . The number  $n$ , therefore, may be expected to be very large compared to  $r$ , the number of hits. If this is true, then

$$(1-a)^{n-r} \stackrel{\text{ca}}{=} (1-a)^n$$

and

$$n(n-1)(n-2)\dots(n-r+1) \stackrel{\text{ca}}{=} n^r$$

and

$$P_r \stackrel{\text{ca}}{=} \frac{1}{r!} a^r n^r (1-a)^n$$

Expanding  $(1-a)^n$  by the binomial theorem gives

$$\begin{aligned} (1-a)^n &= 1 - an + \frac{n(n-1)}{2!} a^2 - \frac{n(n-1)(n-2)}{3!} a^3 + \dots \\ &\stackrel{\text{ca}}{=} 1 - an + \frac{1}{2!} (an)^2 - \frac{1}{3!} (an)^3 + \dots \end{aligned}$$

By MacLaurin's formula this is

$$(1-a)^n \stackrel{\text{ca}}{=} e^{-an}$$

In this way we arrive at the Poisson law<sup>2</sup>

$$P_r = \frac{1}{r!} (an)^r e^{-an} \dots \dots \dots (1)$$

in which  $an$ , the average number of hits, can be calculated from the results of irradiation. If one hit is enough to kill a bacterium, the survival ratios as given

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<sup>2</sup> This expression or its equivalent has been used several times in studies of the effects of X-rays on cells. See for instance Condon, E. U., and Terrill, H. M., *J. Cancer Res.*, 1927, 11, 324; Crowther, J. A., *Proc. Roy. Soc.*, 1926, B 100, 390; Holweck, F., *Compt. rend.*, 1929, 188, 197.



in Table I will be expressed by the probability that a bacterium will be missed every time. As the foregoing considerations have shown, this is

$$(1 - a)^n \stackrel{\text{ca}}{=} e^{-an}$$

In other words

$$\frac{A_1}{A_0} = \text{survival ratio} = e^{-an} \dots \dots \dots (2)$$

If a bacterium could withstand one electron but were destroyed by the second, the survivors would be those which either escaped or were hit once, so that

$$\frac{A_2}{A_0} = e^{-an} + an e^{-an} = e^{-an} (1 + an) \dots \dots \dots (3)$$

Similarly, if  $r$  hits are needed

$$\begin{aligned} \frac{A_r}{A_0} &= e^{-an} + an e^{-an} + \frac{1}{2} (an)^2 e^{-an} + \dots \dots \dots \\ &\dots \dots \frac{1}{r-1} (an)^{r-1} e^{-an} \\ &= e^{-an} \left[ 1 + an + \frac{1}{2} (an)^2 + \dots \frac{1}{r-1} (an)^{r-1} \right] \end{aligned}$$

### Standardization

The average number of hits can readily be calculated from a knowledge of (1) the area of the irradiated surface, (2) the dimensions of a bacterium, (3) the number of electrons striking the irradiated surface and (4) the absorption coefficient of electrons in the bacterium.

The counting of the number of electrons under the conditions of these experiments cannot accurately be carried out.<sup>3</sup> This is due partly to the enormous ionization produced in the air through which a cathode ray beam passes, partly to the fact that the absorption of the radiation from the tube in small thicknesses of matter is not linear and is accompanied by a considerable diffusion of the transmitted beam. Reproducible results which seem to be fairly accurate have, however, been obtained by the use of a counting chamber which consists of a

<sup>3</sup> See also Thaller, R., *Physikal. Zeit.*, 1928, 29, 841.

brass disk enclosed, except for a  $\frac{1}{16}$  inch hole on one side, by a grounded and air-tight brass sheath. This sheath is a cylinder 2 inches in diameter and 1 inch high and is provided with a port for evacuation. The central disk is supported by a thin rod which, mounted in amber, provides electrical connection with one of the galvanometer leads. The current produced by the flow of electrons from the collecting plate to ground through a sensitive galvanometer has been taken to measure the number of electrons in the beam. This outfit has been placed directly behind the cutting tube A of Fig. 1 in the position of the Petri dish during irradiation and counts have been made at the conclusion of individual experiments. In use, grounded shielding tubes protect the emerging electrode of the collecting plate and the galvanometer leads pass through grounded copper sheathing. The shutter and cutting tube were grounded during both the irradiations and the counting measurements. Standardizations have been carried out (1) with the opening free and (2) with it covered by 0.001 inch aluminum foil and with the chamber evacuated. The fact that the two measurements were substantially identical after allowance had been made for absorption in the foil indicates that the potential developed between the collecting plate and the grounded case was insufficient to interfere seriously with the accuracy of measurement by drawing ions from the air in the chamber. The principal errors in the electron counts probably arise, therefore, from the reflection of electrons by the collecting disk and from inhomogeneity in the cathode ray stream over the irradiated area. No effort has been made to introduce corrections for reflection but shifts of the collecting chamber over the area of irradiation indicated that at the distance of the plate from the mouth of the tube, inhomogeneity of beam was unimportant.

Measured galvanometer currents were corrected for the relative areas of the irradiated surface and the opening in the collecting chamber and were expressed in electrostatic units. Taking one E. S. U. as equivalent to  $0.21 \times 10^{10}$  electrons, the numbers of electrons incident per cm.<sup>2</sup> of irradiated surface are recorded in Table I.

The absorption of electrons in the bacteria themselves can scarcely be measured. Because this absorption, like that of X-rays, is not a function of the state of chemical combination it can, however, be estimated from measurements upon cellophane as a substance com-

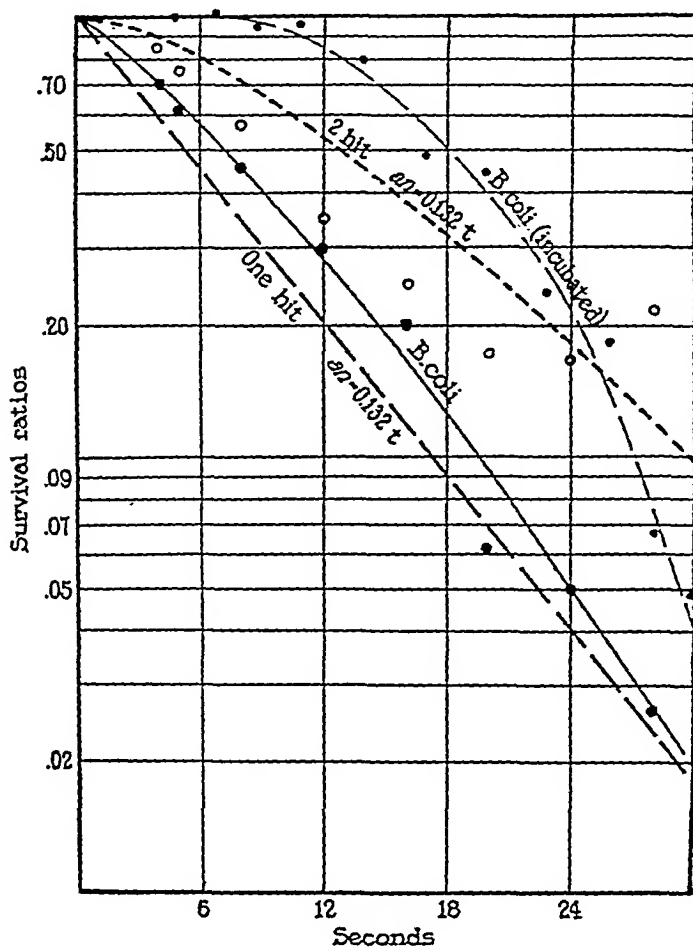
posed only of light atoms and having approximately the density of bacteria. It is well known from studies of  $\beta$ -rays that the absorption of a stream of electrons possessed of different velocities rarely follows any simple law.<sup>4</sup> Measurements of the absorption of the cathode ray beam used in these experiments in successive thicknesses of cellophane lie on a curve intermediate between a straight line and a simple exponential curve. Considering the extrapolation involved in estimating the absorption in a single bacterium from the absorption in a sheet of cellophane (0.0009 inch thick), it has not seemed significant to get more than the order of magnitude of this absorption by a linear interpolation from measureable thicknesses. This procedure has led to the conclusion that there will be an absorption of about 0.008 of the electrons striking either a cylindrical bacterium  $0.5\mu$  in diameter and  $2\mu$  long or a coccus having a radius of  $0.4\mu$ .

#### DISCUSSION

Whether more than one electron is needed to kill a bacterium can be told from the shape of the survival curves. Expression (2), being a simple exponential, will give a straight line as a graph on semilogarithmic paper. Plotted in this way, the equations for multiple hits yield curves which depart further from a straight line the larger the number of requisite absorptions (Text-fig. 1). Some of the data of Table I are shown graphically in Text-figs. 1 and 2. Within experimental limit the results of the best measurements lie on straight lines. From this fact alone it is to be concluded that the absorption of one electron is sufficient to kill a bacterium. The best obtainable data with *Staphylococcus aureus* depart appreciably from a straight line through the origin. This departure is to be expected, however, for microscopic examination shows that even great dilutions contain many clusters of two or more individuals. The effect of a short incubation of *B. coli* between spreading and irradiation is shown by the top curve of Text-fig. 1. Cell multiplication which has taken place gives a curve resembling but more extreme than that from the coccus.

<sup>4</sup> Cf. for instance Rutherford, E., *Radioactive Substances and their Radiations*, London, 1913, Chapter V.

Although one hit kills, it is not necessarily true that every one does so. In Experiment (1) of Table I,  $1.17 \times 10^{10}$  electrons per second



TEXT-FIG. 1. The black circles of this figure are the survival ratios of Experiment (1) of Table I; the open circles refer to Experiment (5) with staphylococci.

are incident upon the irradiated area of  $7.07 \text{ cm.}^2$  The average number of electrons absorbed per second by a cylindrical bacillus  $0.5\mu \times 2\mu$  is then

$$\frac{1.17 \times 10^{10} \times 1.00 \times 10^{-8} \text{ cm.}^2 \times 0.008}{7.07 \text{ cm.}^2} = 0.132$$

and Equation (2) as applied to this experiment becomes

$$\frac{A_1}{A_0} = e^{-0.132 t}$$

The graph of this expression in Text-fig. 1 lies close to, though somewhat below, the experimental survival ratios. The curve of the corresponding equation if two hits are needed to kill (Equation 3) is shown as the thick dotted line of the same figure. Similar relations prevail in the other standardized experiments. The average number

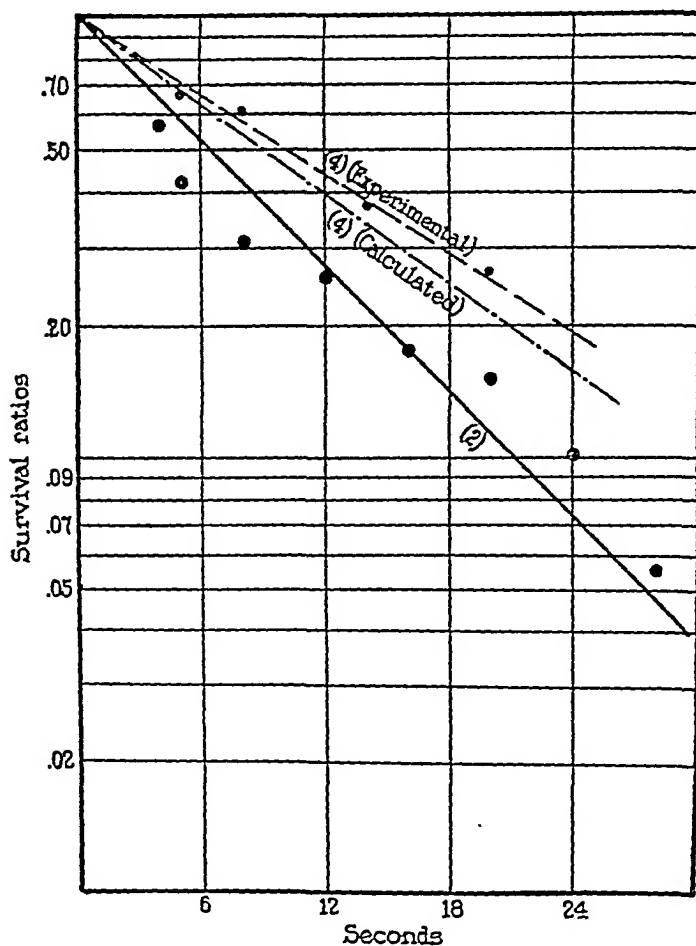
TABLE II  
*Results from Standardized Experiments*

Experiment (Table I)	Electrons absorbed per bacterium per second	
	Observed	Calculated
(1) <i>B. coli</i> .....	ca 0.112	0.132
(3) <i>B. aerl</i> .....	.056	.067
(4) " " .....	.068	.077
(5) <i>S. aureus</i> .....	ca .105	.114

of absorptions per bacterium per second as calculated and observed are listed in Table II. In each instance the theoretical number exceeds the experimental by about 15 per cent. On its face this would mean that only 85 per cent of the bacteria which absorbed electrons were killed, though whenever death was brought about, one electron was sufficient to accomplish it. In view of the inaccuracy of the measurements involved in standardization, however, too much importance cannot be attached to this percentage of killed organisms. It is perhaps safe merely to conclude that the number of bacteria killed is of the same order of magnitude as the number which absorb electrons.

The similarity in the curves for *B. coli* and *B. aertryke* might be expected from their similarity in size and shape. Though none of the experiments using *Staphylococcus aureus* gave results as uniform as with

the two bacilli, the observed greater resistance of this bacterium is adequately accounted for by its smaller size (Table II).



TEXT-FIG. 2. The data of this figure refer to Experiments (2) and (4) with *B. aertryke*.

#### CONCLUSIONS

1. For the two motile bacilli, *B. coli* and *B. aertryke*, the absorption of a single 155 K.V. electron is sufficient to cause death. Furthermore,

all, or nearly all, the electrons absorbed are lethal. The same is undoubtedly true of *Staphylococcus aureus*. In addition to providing a quantitative picture of the interaction of bacteria and cathode rays, these results suggest that radiation of the energy content used in our experiments is not suitable for altering the inheritable characteristics of bacteria.

2. The differences in sensitivity to cathode rays shown by the bacteria studied can be explained by the purely physical factor of size.

3. Counts giving significant conclusions concerning killing rates can be obtained only if there is no clumping of the cells when spread and only if the cells are not allowed to multiply before irradiation. Both these precautions seem rarely to have been met in the experiments that have in the past been made with X-rays and other forms of radiation.

#### EXPLANATION OF PLATE 30

FIG. 1. A photograph showing the anode end of the cathode ray tube and the apparatus used in irradiating the agar plates.

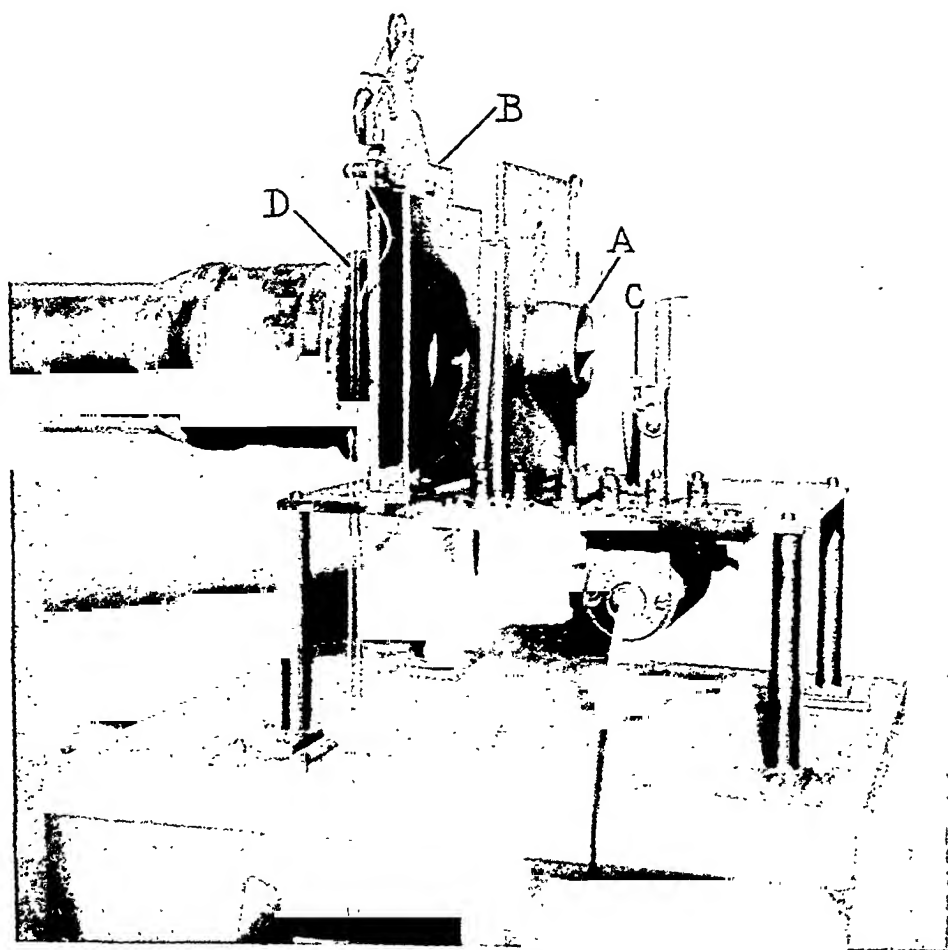


FIG. 1

(Wyckoff and Rivers Effect of cathode rays upon bacteria)





## RISE IN TEMPERATURE PRECEDING THE APPEARANCE OF SYMPTOMS IN EXPERIMENTAL POLIOMYELITIS\*

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(Received for publication, March 31, 1930)

In the main the clinical manifestations and pathological lesions of experimental poliomyelitis in the monkey represent a faithful reproduction of the human disease.

The clinical course of the experimental disease is fairly uniform. The first symptoms vary somewhat in time of appearance. This depends largely on the strain of virus used, the extent to which the virus has been modified either by temperature (1), by chemical agencies (2), or by the addition of convalescent serum (3). When a 7- or 8-day unmodified virus is inoculated intracerebrally the monkey remains normal in appearance (after the effect of the operation and anaesthetic has worn off) until the seventh or eighth day. The first symptoms to appear are tremor of the head and the extremities (more easily noticed in the upper extremities), the ruffled coat and some awkwardness and stumbling when the monkey attempts to climb. Within a few hours these symptoms are followed by paralysis of varying severity and in most instances by complete prostration and death. When the disease does not terminate fatally the animal goes through a convalescent period of several weeks' duration, after which except for the residual paralysis it seems quite normal.

In one respect, however, the experimental disease had been considered as differing from the human form in that a phase of the disease analogous to the preparalytic stage of the human disease was lacking. As is pointed out in this paper this view is not justified. The preparalytic stage of the disease in human beings is now well established

\* This work was supported by the Harvard Infantile Paralysis Commission, a fund privately donated to the Vermont Department of Public Health and a gift from the International Committee for the Study of Infantile Paralysis.

(4), lasting 2 or 3 days before the onset of paralysis and consisting of a syndrome of fever, headache, vomiting, stiff neck and back and a coarse tremor. These symptoms are regularly associated with an increase in the cell count and in the globulin of the spinal fluid. In the experimental disease paralysis follows the earliest symptoms so rapidly that a division comparable to the human preparalytic stage has not previously been often made. On account of the lack of an obviously preparalytic stage there is a question whether certain procedures applied to the inoculated monkey are applicable to the human disease in the preparalytic stage. This uncertainty has been felt particularly in experiments dealing with the effects of convalescent serum therapy on the course of the disease.

In an attempt to determine if there are some earlier detectable manifestations in the experimental disease comparable to the preparalytic stage of the human form, we undertook a series of studies on monkeys inoculated in the course of our work. These studies dealt with the temperature, formed blood elements, spinal fluid, and blood chemistry. Similar studies made on a group of normal monkeys served as controls.

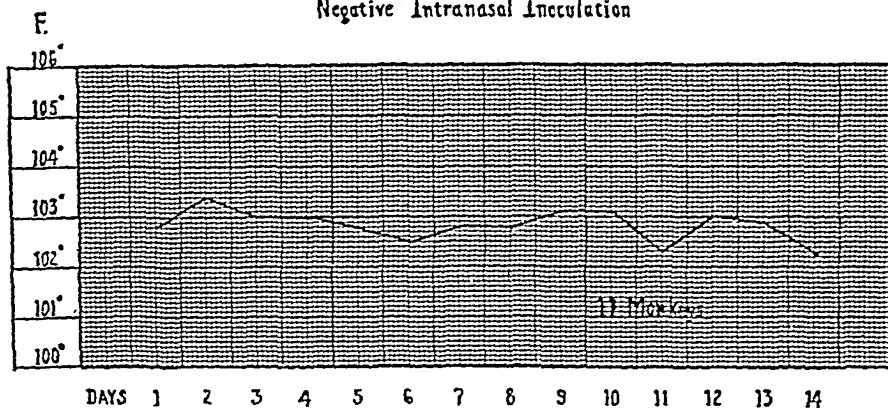
The present communication devotes itself to temperatures observed in normal monkeys and in monkeys infected with the virus of poliomyelitis. Rectal temperatures were used throughout. It was noted early that the administration of ether causes an immediate, though transient, drop in the temperature, so that temperatures were not taken at such times.

The temperature of the normal monkey is about 103°F., varying to some extent, however, without apparent reason in the same monkey both on the same day, and from day to day. Fluctuations of 1° and occasionally 1.5°F. between the maximum and minimum temperatures are not uncommon, but variations of more than 1° from the average mean temperature are rare. The average of 1301 readings on 46 monkeys over a period of several months was 102.9°F. A number of the monkeys showed a temperature consistently higher than 103°F., several ranging between 104° and 105°. The reason for these exceptions was not always clear, although some animals developed diarrhea, and in several instances where the monkeys came to autopsy a generalized tuberculosis was found. In others, however, there was no such explanation.

Graph I is a composite curve of 11 intranasally inoculated animals that resisted infection. The average temperature of 147 readings on

### Experimental Poliomyelitis—Temperature

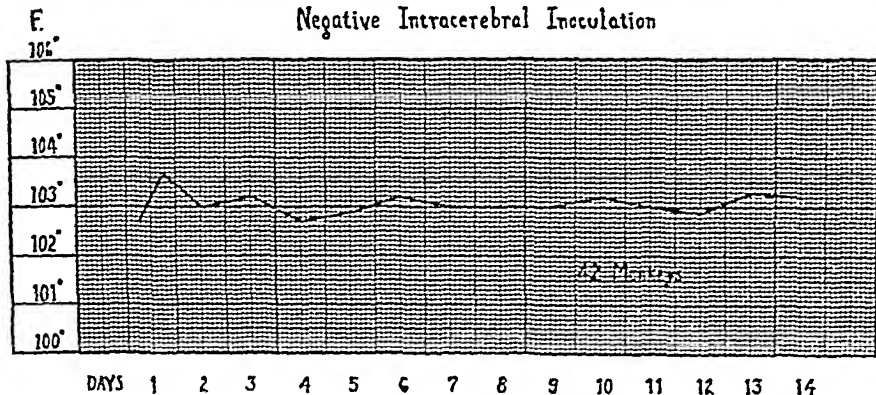
#### Negative Intranasal Inoculation



GRAPH I

### Experimental Poliomyelitis—Temperature

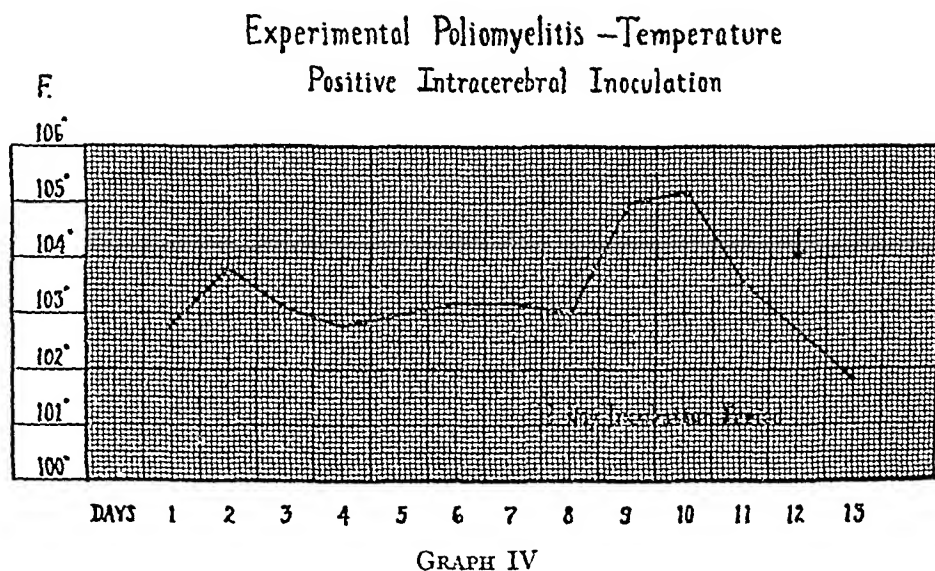
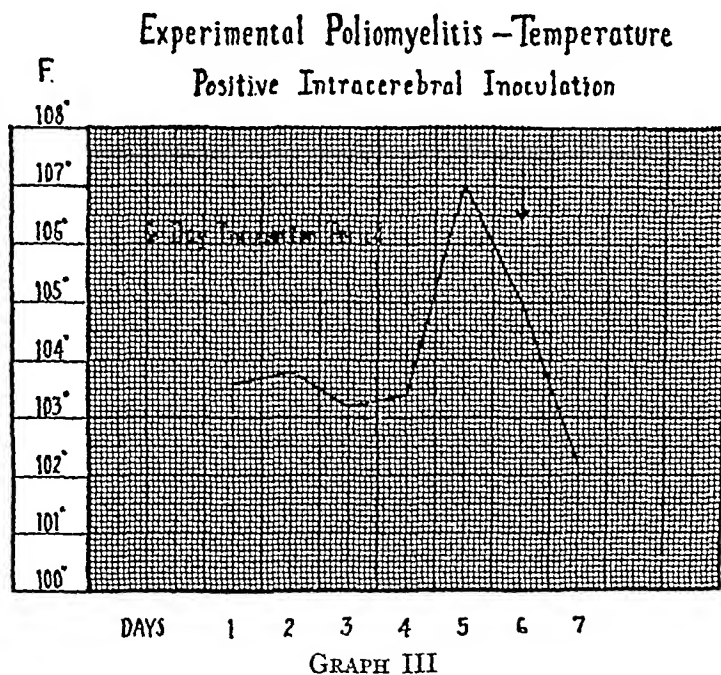
#### Negative Intracerebral Inoculation



GRAPH II

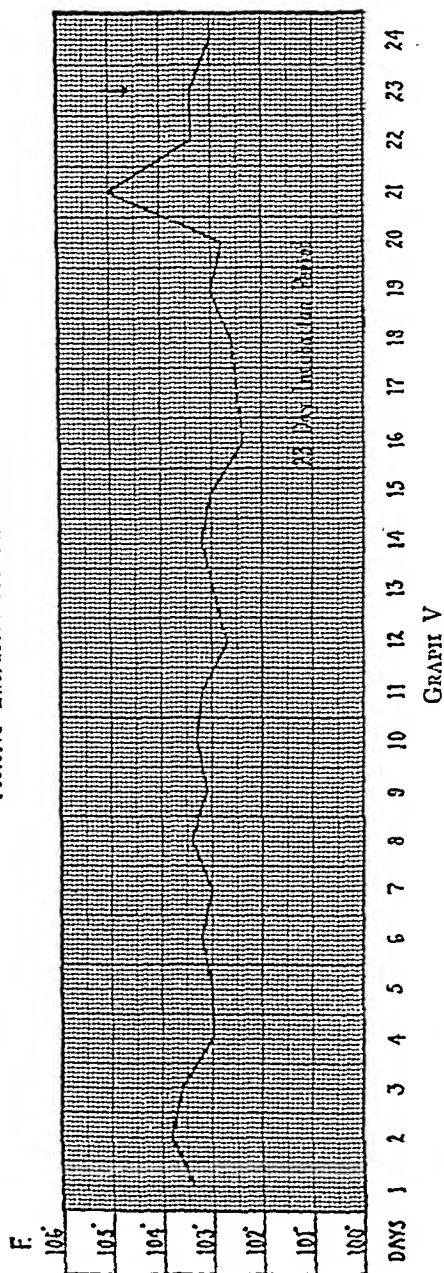
these monkeys was 102.9°F., with a maximum plus deviation of 0.5°F., and a maximum minus deviation of 0.6°F. from the average.

Graph II is a similar composite curve of 42 intracerebrally inoculated animals that did not succumb to the disease. The average tempera-



ture of 470 readings on these monkeys during the "normal" period was  $103.1^{\circ}$ . The maximum plus and minus deviations were  $0.6^{\circ}$  and

Experimental Poliomyelitis — Temperature  
Positive Intracerebral Inoculation

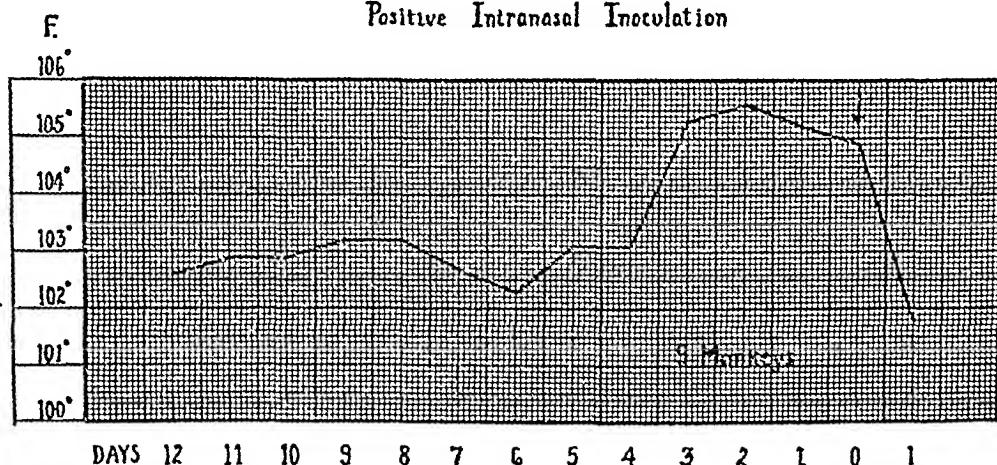


0.4°F. respectively. With the exception of the transient rise following intracerebral inoculation the two curves are identical. The preliminary rise of 1° to 2° following the operation was regularly observed in monkeys inoculated intracerebrally and usually did not last beyond the first 24 hours. This rise was absent in those monkeys in which the virus was instilled into the nostrils.

The temperatures of the monkeys which succumbed, however, showed constant and characteristic alterations from the normal. Graphs III,\* IV, and V show the temperature curves of a number of intracerebrally inoculated animals that developed the disease. These

### Experimental Poliomyelitis—Temperature

#### Positive Intranasal Inoculation

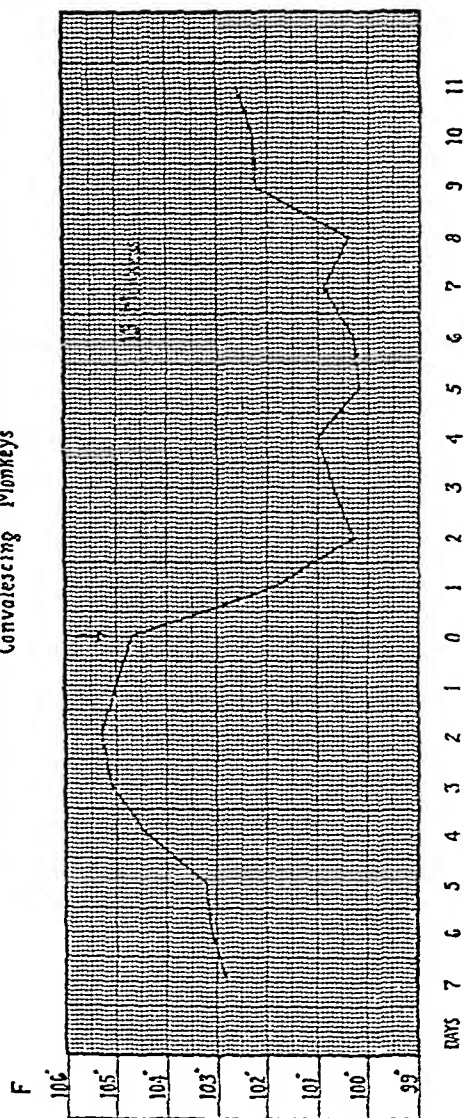


GRAPH VI

graphs show the preliminary rise, already referred to, followed by a varying period of "normal" temperature, followed in turn by an abrupt rise which reaches its maximum about 24 hours before the onset of the earliest symptoms. This interval of "normal" temperature has at times lasted 18 to 21 days, as in the instances of prolonged incubation period illustrated in Graph V. This type of curve occurred in about two-thirds of the animals that succumbed. In the remaining animals, however, the second rise was more gradual, although the temperature, as in the other animals, reached its maximum about 24 hours before the onset of symptoms.

\* On this and subsequent charts the date of appearance of symptoms is indicated by an arrow.

Experimental Poliomyelitis - Temperature  
Convalescing Monkeys



GRAPH VII



Graph VI\* is a composite temperature curve of 9 monkeys successfully inoculated intranasally. These animals did not show the early preliminary rise noted in Graphs III, IV, and V, but the temperature rise preceding the manifestation of symptoms appeared in a manner identical to that observed in the intracerebrally inoculated animals.

Coincident with this preparalytic rise in temperature, spinal fluid changes were observed similar to those in preparalytic human poliomyelitis. Details of these findings will be published in a later communication.

The trend of the temperature curve after the monkey is paralyzed and prostrated is illustrated in Graph VII.\* With the appearance of the usual symptoms of the experimental disease the temperature is already declining. Within 24 hours after the appearance of paralysis it has usually become subnormal. When paralysis is extensive and the prostration marked the temperature has in several instances dropped as much as 10°F. That this pronounced drop in temperature is due in part to external factors is indicated by the fact that it was possible to avoid such an extreme drop by keeping the animal warm. The return of the temperature to the normal range in surviving monkeys seemed to depend considerably on the degree of prostration as well as the temperature of the environment. Several such monkeys were carefully nursed for a number of weeks and Graph VII shows the gradual return of the temperature to the normal range as the monkey's general condition improved.

#### SUMMARY

The data presented in this paper offer a means of earlier recognition of experimental poliomyelitis in the monkey. The early appearance of the temperature rise (1 to 3 days before the onset of the usual recognizable symptoms) associated with spinal fluid changes suggests that there is a stage in the experimental disease corresponding to preparalytic human poliomyelitis.

\* Since some of these animals had been given repeated intranasal instillations of the virus, the incubation period was not ascertainable. Hence, the temperature readings are expressed in days preceding the onset of symptoms.

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# ABNORMALITIES PRODUCED IN THE CENTRAL NERVOUS SYSTEM BY ELECTRICAL INJURIES\*

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PLATES 31 TO 33

(Received for publication, February 27, 1930)

The problem of injuries following contact with electric circuits has become an important one owing to the extensive use of electricity in everyday life. In 1913, 670 deaths in the United States were attributed to this cause. Circuits supplying homes and factories are commonly of 110 or 220 volts, either alternating or continuous current. A 100 volt circuit may prove fatal. For this reason it has seemed important to make a careful fundamental study of electrical injuries with a view to saving the lives of those who have received a severe shock. The present paper presents experimental evidence concerning the injuries produced in the central nervous system.

The material serving as a basis for this study consists of some 286 rats, injured by either continuous or alternating electric circuits at different potentials. The experimental technique and the immediate results of the injury have been reported in another place (Langworthy and Kouwenhoven, 1930). The central nervous systems of these rats were preserved immediately after death and typical sections have been cut in an endeavor to correlate the experimental results with actual histological pictures. It will be possible to show that the abnormalities produced by the continuous current differ from those produced by the alternating.

Many fatal accidents from contact with electricity are attributed to the resulting fibrillation of the ventricles of the heart. Ventricular fibrillation is believed to be more common after contact with circuits

\* This research was conducted with a fund provided by the Committee on Electric Shock.

at low voltages. Circuits at high voltages, on the other hand, are thought to cause death from respiratory failure due to a central inhibition in the nervous system. Since the present experiments were directed to an elucidation of injury in the brain and spinal cord, an experimental animal was chosen in which the heart recovers spontaneously from ventricular fibrillation. The rat proved to be such an experimental animal; it is doubtful if any deaths in this series can be attributed to cardiac failure. This makes it possible to focus attention upon the central nervous system.

The histological study reported here endeavors to demonstrate evidence of injury in the nerve cell itself as a result of electric shock. These changes are sought in the general appearance of the cell, in the Nissl granules and in the cell nucleus. These criteria of injury of the nerve cell have been used by many investigators.

A good review of the pathological changes produced in nerve cells by poisons, such as arsenic, phosphorus and veratrin, by anemia and by high temperatures was given by Barker (1909). It has been demonstrated that the Nissl granules are formed after death by the coagulation of some substance normally present in the cell. The substance is used in cell metabolism and is greatly decreased in amount by fatigue. As the result of injury, various changes are observed in the cytoplasm; among these may be suggested complete loss of granules, loss of granules at the periphery of the cell or around the nucleus and vacuolization. The cell may be swollen, the granules large and adherent in a compact mass or the cytoplasm stain a uniform blue. Later a contraction of the cytoplasm and pyknotic condition of the nucleus suggest cell death. Buzzard and Greenfield (1925) have more recently reviewed the subject of nerve cell pathology. Here it is important to discuss the changes that have been found in nerve cells following injuries with electric currents and closely allied types of traumata.

Urquhart (1927) felt that the asphyxia accompanying temporary respiratory inhibition was sufficient to produce changes in the cells of the brain and cord that would confuse any picture of injury produced by electricity.

Gomez and Pike (1909) previously found that anemia lasting 8 minutes was sufficient to kill the small pyramidal cells of the cortex. After 13 minutes there were marked changes in the Purkinje cells in the cerebellum. In the case of the medulla, anemia for 20 to 30 minutes produced alterations incompatible with complete recovery. Cannon and Burket (1913) later showed that cells of the myenteric plexus could recover from anemia lasting 6 hours. In the present experiments the periods of apnea were short; the rats that were saved breathed in almost every case within 2 minutes. It seems that the effects upon the nerve cells of anemia may be discounted in the present work.

Mott and Uno (1922) studied the central nervous system of patients that died from surgical shock from 24 to 48 hours after injury. The brains were fixed first in formalin and afterward blocks were placed in alcohol. In the cerebellum very few Purkinje cells showed a normal staining reaction. The Nissl granules were broken up into fine dust; the cell was swollen, the nucleus indistinct and the whole cell only faintly stained. In the neighborhood one could see cells which presented a more normal appearance.

Regarding the changes produced in the nerve cell by electrical injuries, observers do not agree. Small perivascular hemorrhages have been reported, especially in the medulla and floor of the fourth ventricle; these hemorrhages are common after legal electrocution. Chromatolysis of the ganglion cells and rupture of the cells with dislocation of the nuclei have been seen. Huber described a loosening of the glia, vacuolization of the nerve cells and approximation of the nuclei to the walls of the cells. Kawamura says that some cells appear normal while others are shrunken and hyaline. In the medulla oblongata the Nissl substance is broken into small granules. Mott and Schuster (1909) described the lesions in a man who recovered from a shock at 20,000 volt potential and lived for 7 hours thereafter. The autopsy was performed 39 hours after death.

Spitzka and Radasch (1912) sectioned the brains of five electrocuted criminals and found peculiar areas from 25 to 200 micra in diameter around small blood vessels. There was a central rarified zone and a peripheral condensed one. They explained their observations on the basis of a sudden liberation of gas bubbles by the electrolytic action of the current. Since the current used was alternating, it is more likely that the vacuolization resulted from excessive temperatures. In electrocution the brain becomes exceedingly warm, a temperature of 145°F. has been recorded.

MacMahon (1929) has performed experiments similar to those reported in the present paper. Guinea pigs were exposed to continuous circuits of low voltage. Some of the animals received a series of shocks on different days. Following the injury, excitability, nervousness and stupor were observed. There was also weakness, paralysis of the posterior extremities and diminished sensation. The animals were less active and did not move about quickly after the shock. Paralysis of both posterior extremities occurred in more than half the animals. In some cases it came on immediately after the shock; others did not become paralyzed until 2 days thereafter. When paralyzed, the animals dragged their hind feet and appeared to have no control of the sphincters. If the paralyzed legs were lightly stimulated by touch, pressure, pain or heat there was no response.

On examination of the central nervous system of the guinea pigs that had survived the shock it was found that the spinal cord was swollen, soft and almost diffuent. There were petechial hemorrhages in the pia arachnoid. The white matter of the cord was most seriously injured, and the lesion was greatest in the posterior columns. The earliest change in the nerve fiber consisted in a swelling of both the axis cylinder and myelin sheath. The myelin in later stages disappeared

leaving irregular vacuoles and spaces around and among the swollen axones. The peripheral nerves showed similar degeneration. Little information was given concerning changes in the nerve cells.

With this review of the literature, it is important to discuss the peculiar difficulties in interpretation of pathological changes in nerve cells as seen in sections stained with a basic dye. Criteria that are used by investigators as evidence of nerve cell damage have already been presented. But, in order that these criteria may be valid, other very definite postulates have to be filled.

To demonstrate the finer structure of cells, and in particular, nerve cells, it is extremely important that fixation of the tissue be carried out immediately after death. If the autopsy is delayed for only a few hours, the nerve cells stain a uniform, blue color with the basic dyes. Moreover the fixative is important; 95 per cent alcohol should be used if a sharp, clear staining of the granules in the cytoplasm is desired. Formalin is a standard fixative for nervous tissues but it permits only a poor differentiation of the granules. The 95 per cent alcohol has a very low power of penetration; with fairly large brains an arterial injection is important. The central nervous system of the rat is so small that the brain was not injected but was cut in thin slices and immersed in the fixative.

It is often difficult to obtain a good staining of the sections. A good thionin dye becomes slightly metachromatic and imparts to the granules a deep blue tint with a trace of lavender. The sections with this lavender hue do not fade easily and remain in good condition indefinitely. If the sections are overstained the cytoplasm of the cells is a uniform blue and the granules are not distinct; if the stain is light the granules do not receive their full value.

After a sharp staining of the cells, their nuclei and the granules in the cytoplasm is obtained, the interpretation still offers difficulties. Different groups of cells show great variation as to their size, shape and the arrangement of the granules. Characteristic patterns of the granules have been studied by Malone (1912 and 1913). He pointed out that somatic motor cells have large Nissl granules packing the cytoplasm of the cell. Autonomic cells, on the other hand, have large granules surrounding the edge of the cytoplasm and smaller granules in the remaining area. Sensory cells have fine, dust-like granules, scattered uniformly. The possibilities of granule arrangements in the higher centers of the brain are infinite.

To avoid difficulties of telling normal from injured cells by the arrangement of granules, only well known types of cells are discussed in the present report. The somatic motor cells of the anterior columns of the cord were examined in every case. It was most important to study the dorsal nucleus of the vagus because of its important func-

tion in respiration. The Purkinje cells of the cerebellum are characteristic as are the cells of the olives. Finally the sensory cells in the mesencephalic nucleus of the trigeminal were used as a type. No minute examination of the cells of the cerebral cortex was made. The sections of the forebrain were examined for hemorrhage and evidence of burning, as was the rest of the brain and cord.

### *Methods*

The brain and spinal cord of the rats that died as an immediate result of the injury, were removed at once, cut into thin slices and placed in 95 per cent alcohol. After adequate fixation the blocks were imbedded in paraffin and sections stained with thionin. Rats that survived the shock were killed a few hours or days after the injury. In the case of a number of the rats, a microscopic examination of the thoracic and abdominal organs was made.

### HISTOLOGICAL STUDY

The experiments are grouped on the basis of different potentials of alternating and continuous circuits. Greater injury was produced by the circuits at high voltages and these marked changes may be described first.

#### *Rats Subjected to a Continuous Circuit at 1000 Volt Potential*

It will be possible to demonstrate that the general microscopic picture of injuries produced with the continuous circuit is somewhat different from that produced by the alternating. Since the lesions are more severe and more clear cut with the 1000 volt circuit it is proposed to begin with this group.

Thirty-three rats were subjected to a continuous circuit at 1000 volt potential for periods from quick manual closure and opening of the switch to 4 seconds. For the purpose of study a contact of  $\frac{1}{4}$  second was approximated as closely as possible. The current readings were 600 to 1100 milliamperes. Twenty of the rats died at once, nine recovered and four were paralyzed. These findings need further explanation; thus eight received the current for the time required to open and close the switch. Of these eight animals, five recovered, two died and one was paralyzed. Thirteen were shocked for approximately  $\frac{1}{2}$  second; four were normal, three paralyzed and six died at once. No rats survived an application of the current for 1 second or over.

By comparison it is found that the continuous 1000 volt circuit was more injurious to rats than the alternating circuit of the same potential.



This is particularly true if the time element is taken into consideration. But, at autopsy, few gross hemorrhages were found in the central nervous system, even of the paralyzed rats. Study of the sections gives an explanation for the fatalities in this group.

Twenty rats that died immediately will be considered first, in reference to the microscopic findings.

The photomicrographs of abnormal cells that illustrate this paper were made largely from sections of the central nervous system of this 1000 volt group. Large numbers of sections are available and the pathological picture is remarkably uniform. In three of these rats there was a large hemorrhage in the fourth ventricle that must have pressed upon the respiratory center. The brains, in general, were remarkably free from microscopic hemorrhages.

The surface of the cerebral cortex was not as severely injured as in the alternating circuit series. It is true that in several cases large cavities were found near the surface of the gray matter. These have been observed in the brains of electrocuted criminals and are supposed to be due to the collection of gases produced by the generation of heat. It is interesting that in the rats they are found most commonly close to the surface electrode where the concentration of electricity is great, although they do occur in all portions of the nervous system. They are often present in rats that recovered from the shock. In man they are perivascular; this is not necessarily true in the rats. The wall of these cysts is deeply staining due to the condensed brain tissue. In some of the rats the surface of the cortex appears to have actually burned and the remaining edge stains an intense blue.

The greatest interest centers around the changes in the nerve cells that are similar to those produced by the 1000 volt alternating circuit, but more severe. These may be described first in the somatic motor cells of the spinal cord.

The cells are selectively injured; some are grossly damaged and others appear almost normal. The cell in Fig. 4 from a rat of this series has a nucleus that appears practically normal. On one side of the cytoplasm the granules are numerous. On the other, toward the periphery of the cord, few granules are present. The cytoplasm appears shrunken and the outlines of the cell can be made out with difficulty. Since this cell has a normal nucleus it is thought that it has the potentialities of a complete recovery.

In Fig. 5 the cell presents a somewhat different picture. The nucleus stains deeply and the nucleolus is swollen but the nuclear injury is scarcely profound. Granules are numerous around the nucleus but the outer portion of the cytoplasm is relatively free from granules. This cell has suffered a loss of Nissl granules.

A severely injured cell is shown in Fig. 6; this opinion is given because of the

nucleus which is shrunken, and stains a uniform deep color in which no nucleolus or chromatin skeins can be seen. The clear area around the nucleus shows its normal size. The granules are numerous in this cell, although portions of the cytoplasm are quite free from them.

In Fig. 7 are two cells with nuclei slightly less damaged. The nucleolus is greatly swollen. The remainder of the nucleus is shrunken and darkly staining although the color is not as deep as the nucleolus. In the upper cell the granules are sparse but scattered uniformly. The cytoplasm has no distinct boundaries in the lower cell and it would appear that the cell has ruptured and the granules are free in the perivascular space. It is doubtful whether these cells have the potential power of recovery.

The deep staining cell in Fig. 8 is similar to that in Fig. 6; the other two cells show severe damage. The nucleus in the lower one, however, appears quite normal. The cytoplasm of both these cells is greatly shrunken and the edges are jagged and irregular. If the nucleus is normal it is probable that the cytoplasm may be regenerated. Similar cells are abundant in all this material and suggest the severe injury responsible for the death of the rats.

The Purkinje cells are particularly vulnerable to injury with electric shocks.

The results of the shock, as seen in the rats that die immediately, are seen in Fig. 12. The injury is again selective although large groups of cells are abnormal as the photomicrograph suggests. The nuclei of the cells stain a uniform, deep color in which the chromatin and nucleolus cannot be distinguished. The cytoplasm likewise stains a lighter but uniform blue. That this injury betokens the death of the cell is clear from the examination of rats that have survived for some time (Fig. 11). Here the cells stain a dark color and are much smaller than normal.

Since sensory ganglia were not stained in every preparation, the cells of the mesencephalic nucleus of the trigeminal were used for illustrating damage to primary sensory neurones.

The nucleus of the cell (Fig. 16) is irregular in outline and small but the nucleolus may still be distinguished. The clear surrounding area was probably filled by the nucleus. The granules show poorly in the cytoplasm which stains a rather uniform blue. These cells are severely damaged. Fig. 15 shows several fairly normal cells and one with a deep staining, pyknotic nucleus. Sensory cells are relatively resistant to injuries by electricity.

It was considered particularly important, in each case, to examine the dorsal nucleus of the vagus since the cells of this nucleus have an important part in initiating and controlling respiration.

These autonomic cells normally have large granules at the periphery of the cytoplasm and smaller granules scattered through the remainder. A fairly typical illustration of the granules is seen in Fig. 17. However, numbers of the cells in these rats, as shown in Fig. 17, have deep staining nuclei suggesting the death of the cells. One of the cells in Fig. 18 has a deep staining nucleus; in another the edges of the cytoplasm are indistinct and a loss of granules has occurred. More severe injury is seen in Fig. 22 where both nuclei and cytoplasm are grossly injured. If many cells of the nucleus are thus damaged not a temporary block but a permanent paralysis of the respiratory center is to be expected.

Four rats were paralyzed following the shock. There was a tendency toward recovery so that the legs became much stronger in one of the preparations and by the fourth day they were used fairly well. Nocuous stimuli applied to the paralyzed portion of the body elicited a response in all of these rats, in contrast with the alternating group. Two of them became incontinent on the second day, although no blood was ever present in the urine. All the alternating group were incontinent and the urine was deeply stained with blood. Moreover, priapism was present in these rats; this suggested an irritative lesion in the spinal cord. Macroscopic examination of the brain and spinal cord revealed no lesions that would account for the marked symptoms.

These rats lived from 2 to 6 days following the shock. On microscopic examination of the cord it was found that many ventral horn cells had recovered from the initial injury and were fairly normal in appearance. The granules, however, were not well defined and the cytoplasm tended to stain a uniform deep blue. Other groups of cells were present which showed practically no granules in the cytoplasm. The third type of injury, found particularly in the lumbar portion of the spinal cord was the most serious and characteristic; it is represented in Fig. 9. The cells are deeply staining and the cytoplasm has irregular contours. The nucleus is distinguished with difficulty; in the upper cells and the one on the lower right, the nucleolus may be recognized, staining a little deeper color than the cytoplasm. The cytoplasm is very shrunken. The two lower cells are being attacked by phagocytes that have penetrated the cytoplasm of the cell. That this is not the nucleus is clear in the lower right hand cell where both the nucleolus of the nerve cell and the phagocytic cell may be distinguished. This phagocytosis is evidence of severe cell injury and death. We have, then, in this group, paralysis of the posterior

portion of the body, not due secondarily to the influence of a large hemorrhage upon the cells and fibers of the cord but to an actual injury of the nerve cells. This injury is not as widespread as in the cases of hemorrhage and only groups of nerve cells are injured. It is important to speculate as to whether the hemorrhage was the sole factor producing the paralysis in the alternating circuit series. Since the tracts were not severely injured in the present group sensation was not abnormal. Irritation of the autonomic cells of the cord produced the priapism and severe damage of similar cells was responsible for the incontinence. The atony of the bladder musculature was not great enough to produce hemorrhages and subsequent hematuria. As the cells tended, to some extent, to recover their functions, the abnormalities became, in some cases, less marked.

Nine of the rats recovered and lived for periods from 5 hours to 3 days. There is considerable infiltration of the ventral columns of the cord with phagocytic cells. The motor cells, in general, stain deeply 5 hours after the injury but the Nissl granules are poorly defined even after a period of days. Many cells are necrotic.

The injury of the Purkinje cells in the group that survived may be seen in Fig. 11. The severely injured cells become shrunken and pyknotic, forming a conspicuous group, contrasting sharply with the normal cells. Others (Fig. 13) not as severely injured show large vacuoles in the cytoplasm. Fig. 14 again demonstrates the dead cells. Large numbers of Purkinje cells are injured and the damage is greatest on the dorsal surface of the cerebellum.

Fig. 21 is a photograph of the dorsal nucleus of the vagus from the brain of a rat surviving the shock for 4 days. The shrunken, pyknotic, dead cells are sharply contrasted with the normal autonomic cells. A somewhat different pathological picture in the dorsal nucleus of the vagus is given in Fig. 20. At the lower edge is a fairly normal cell and at the upper edge a shrunken pyknotic one. Between are two cells that have completely disintegrated.

Fig. 19 shows the injury as observed in the superior olive of a rat that survived the injury. Contrasted with normal cells are others with deeply staining cytoplasm and nucleus.

#### *Rats Subjected to Continuous Circuits at 500, 220 and 110 Volt Potential*

The histological findings in groups subjected to these voltages will be summarized together. Only one type of abnormality was observed as an immediate result of the injury that has not been mentioned in

connection with the 1000 volt circuit group. Five rats subjected to a continuous circuit at 500 volts exhibited typical clonic convulsive seizures, some 5 or 10 minutes after the shock. These convulsive attacks occurred spontaneously or upon touching the rats, which were definitely hyperirritable. They never continued longer than an hour and the rats were perfectly normal thereafter. No gross lesions were demonstrable in the central nervous system. No histological change was found to explain the convulsions; it is true that these animals lived for some time after the injury and after the convulsions had ceased. The outer portion of the cytoplasm of many of the ventral horn cells was relatively free from granules. No lesion was found in the vestibular nuclei.

The cellular injury in the rats that died immediately was much less severe after shocks with the lower voltages. In like manner, recovery of the abnormal cells was more rapid. Some trauma to the Purkinje cells was observed with the longer applications of the current. There was in every case a definite diminution of the Nissl granules. The impression is gained that prolonged artificial ventilation of the lungs might have saved many of these animals.

#### *Rats Subjected to an Alternating Circuit at 1000 Volt Potential*

Twenty-eight rats were exposed to an alternating circuit at 1000 volt potential; only one received the current for 4 seconds and the remainder for 1 to 3 seconds. The current produced a severe injury at the site of the electrodes; in the case of the head electrode there was often a burning of the brain. Of the 28 rats, 11 were paralyzed; 9 died at once and 8 were normal.

At autopsy ten of the eleven paralyzed rats showed a typical hemorrhagic lesion of the spinal cord that could be recognized grossly. They all died within 3 days after the injury. Sections showed a similar hemorrhage in the eleventh so that it may be assumed that paralysis in each case was due to a hemorrhage into the spinal cord.

These hemorrhages occurred frequently in the rats shocked with alternating circuits at all voltages. The bleeding occurred most commonly in the lower thoracic region, the hemorrhage extending in both a cranial and caudal direction. It was most commonly produced by the rupture of the posterior spinal artery and from this point the blood spread out into the posterior columns (Fig. 1). Several

rats that appeared normal during life proved to have a localized injury of this type. With further extension, the blood broke into the central canal of the cord which permitted a greater spread. The anterior horn cells and nerve fibers of the ventro-lateral columns were frequently pressed upon and paralysis ensued. In many cases the spinal cord was necrotic in the thoracic region (Fig. 2), indicating a complete transverse lesion. It is interesting that a spinal ganglion beside a completely necrotic area of spinal cord showed little change in the cells. The cytoplasm of these cells was pale and contained few granules.

The hemorrhage did not always involve the posterior column fibers first; other hemorrhages were observed in the ventro-lateral columns. It was rather common to find blood bilaterally in the ventral columns due to the rupture of the small arteries that travel with the ventral roots. The sensory pathways were injured so that nocuous stimuli applied to the posterior portion of the body elicited no response. The rats were in every case incontinent and, after a few hours, large amounts of blood appeared in the urine.

At autopsy it was found that the bladder was filled with blood clot. The atonic bladder had become so enormously distended with urine that hemorrhages had occurred in the wall and blood escaped into the urine. The paralyzed rats were never active and never ate following the shock. They could only be kept alive for relatively short periods.

The mechanical pressure of the blood had a severe effect upon the motor cells of the cord; this is shown in Fig. 3. The granules in the cells are very sparse. In the largest cell there are a few granules around the nucleus but the outer portion of the cytoplasm is quite free from them. The nucleus of the large cell is displaced toward one edge of the cytoplasm.

Concerning the mechanism of the hemorrhage, one possible theory is this. During the passage of the current an extremely powerful contraction of all the musculature of the body occurs. This is strong enough to tear the muscle fibers and frequent hemorrhages are found, for example, in the deep back musculature. The sudden flexion of the back that occurs when the current is applied may be sufficient to cause a rupture of small arteries in the cord. With a continuous circuit the contractions of the musculature are not as strong and the hemorrhages in the nervous system are few. The hemorrhage may, on the other hand, be attributed to venous stasis during the shock and the high blood pressure that immediately follows the injury.

Developing hemorrhages in the spinal cord were found in four of the rats that died as an immediate result of the shock. Likewise small hemorrhages, involving the posterior columns of the cord only, were present in two of the rats that appeared perfectly normal after the injury.

In all of the rats subjected to the alternating circuit at this potential, there was some injury of the dorsal surface of the brain, due to burning in the region of the head electrode.

This injury was severe over the cerebral cortex. There was a superficial loss of gray matter and the remaining edge was left as a deeply staining seared area. In several of the rats that survived an abscess occurred in this area. Large circular holes occurred just beneath the surface of the cortex without relation to the blood vessels. These are thought to be produced by the great generation of heat. The surface of the cerebellar cortex was, in many cases, injured in a similar manner and the cell injury was greatest close to the surface. In the brain stem there were clear, unstained areas surrounding the small blood vessels. These vacuolated areas have been described in the brains of electrocuted criminals.

The changes in the nerve cells may be described first as they were seen in the nine rats that died as an immediate result of the shock.

The abnormalities are marked and increase in severity in direct ratio with the time that the current is allowed to flow. After a 3 second application the granules in the ventral horn cells are greatly decreased in number. Fig. 4 may be taken as an example of such a cell although the animal was injured with a continuous circuit. The cytoplasm is shrunken and the irregular outlines of the cell are enclosed by a dilated perivascular space. Often it appears that the cell membrane has ruptured and the granules escaped.

The histological appearance of a group of cells, for example the ventral horn cells, is not uniform in a section. While the previous description holds for many of them, others appear shrunken and more deeply stained than normal. In these deep staining cells the granules can no longer be seen and the whole cytoplasm takes the deep blue color. Others cells appear almost normal in appearance. It is clear, therefore, that the nerve cells of the same group are not injured to an equal amount by the electric current; some are killed, some are injured and others are practically normal.

Perhaps the most important information concerning the extent of the cell injury is obtained from an examination of the cell nucleus. This has been illustrated by the photomicrographs from sections of rats injured with the continuous currents.

Thus the cell in Fig. 4 has a relatively normal nucleus with the chromatin arranged in finer skeins and the nucleolus sharply defined. In two cells in Fig. 3, normal nuclei are seen. They appear in the cells as relatively clear areas in a cytoplasm packed with deeply staining granules. In Fig. 7, showing cells severely injured by the current, the nuclei have a different appearance. The whole nucleus

is shrunken and takes a uniform bluish stain with scattered, deeper staining chromatin strands. It contains a swollen nucleolus. In the lower of the two cells in the photograph, the edges of the nucleus are irregular in outline.

The nerve cell in Fig. 6 is even more severely injured. The nucleus is greatly shrunken and is surrounded by a white, unstained area which is free from granules. The whole nucleus stains a uniform bluish color in which chromatin and nucleolus cannot be distinguished. A similar condition is seen in the darkly staining cell in Fig. 8.

From a study of this material, it has been concluded that a prognosis concerning the future recovery or death of a cell can be given from examination of the nucleus. If the nucleus appears normal, it is to be expected that the cell will recover its normal structure and function, no matter how severe the injury of the cytoplasm and granules. Thus, it is thought that the damaged cell in the lower portion of Fig. 8 has a good chance of recovery. On the other hand, a uniform staining, dark, shrunken nucleus betokens a cell that has no potentialities for recovery. Further support for this theory will be given in later pages of this report.

It has already been suggested that histological examination of the nerve cells from the central nervous system of rats that died as an immediate result of the shock shows that not all the cells were equally damaged. A whole range of variation may be observed in one single group. This variation may be due in some way to differences in the chemical composition or relative fatigue of these different cells. It may well be that death or functional damage following the shock is proportionate to the number of cells severely injured.

Changes similar to those described for the somatic motor cells in the cord are found in the brain stem and in particular, in the dorsal nucleus of the vagus. A slight damage of cell groups in the medulla might account for temporary respiratory block; more severe injury means the death of the rat.

The Purkinje cells of the cerebellum are easily injured by toxic poisons, by anemia and similarly by the electric currents. In sections from normal animals, it is frequently observed that a few of these cells are shrunken and pyknotic. The injury is so much more extensive in the present series that there can be no doubt as to its pathological significance.



These changes in the Purkinje cells are more marked in the rats that survived the shock by hours and days and less clearly demonstrable in those that died following the injury. Fig. 10 shows normal Purkinje cells and the light staining, large cells in Fig. 11 are even more characteristic. The cytoplasm contains fine scattered granules that give a foam-like appearance to the cell. In the rats that died, following the shock, the nucleus tends to stain a rather uniform blue color in which the chromatin and nucleolus can still be distinguished (Fig. 12). The granules in the cytoplasm are indistinct and the whole cell stains a light blue.

The animals that recovered were allowed to live for various periods of time; signs of recovery of the cells are clearly seen in the sections.

One rat died at the end of 6 hours. The number of cells with blue staining nuclei suggests that the cell damage had been profound. Even at this time the picture is different from that immediately following the shock. Certain cells have acquired a deeper staining of the cytoplasm, although it does not appear as distinct granules but, rather, as a uniform blue color. This is most clearly seen in the Purkinje cells. The latter are always more severely injured close to the dorsal surface of the cerebellum where the concentration of current was doubtless greatest.

Rats that have survived 2 and 4 days show further repair of the nerve cells.

The anterior horn cells at the end of 2 days are still shrunken and surrounded by a large perivascular space. The cytoplasm stains deeply and the granules are beginning to stand out clearly. Many of the nuclei have a bluish tint and the nucleolus is swollen. Other cells have made no recovery and appear to be disintegrating. After 4 days the cells are not yet completely normal in the structure and arrangement of granules in the cytoplasm.

Repair seems to be slower in the brain stem so that even at the end of 4 days many cells in the dorsal vagus nucleus contain few granules in a cytoplasm that is distinctly vacuolated. Here, deeply staining, dead cells may also be seen.

The picture in the Purkinje cells of the cerebellum is very characteristic at this stage and is represented in Fig. 11. Numbers of the cells, scattered among those of normal appearance, are shrunken and pyknotic, staining an intense color. These cells are found everywhere but are more common near the dorsal surface of the cerebellum. Where many of them are dead there appears to be a great disintegration of nerve fibers so that the Purkinje cell layer is easily pulled away from the granular layer, leaving a marked fissure in the sections.

The histological picture of the nerve cells in the paralyzed rats corresponds closely in general to that described for those that appeared normal when killed.

Due to the hemorrhage and subsequent degeneration of nerve fibers, the cord and brain stem were found to be markedly soft when removed at autopsy. In the completely necrotic portions of the cord the nerve cells are of course completely disintegrated; in other areas they are compressed by the hemorrhage producing characteristic changes. The nucleus is displaced to the periphery of the cytoplasm and there is a distinct loss of granules particularly in the periphery of the cytoplasm. In the areas of hemorrhage numbers of white blood cells are found, a majority of them polymorphonuclear. They wander into the gray matter around the injured cells.

The nerve cells in the brain stem make a slow and incomplete recovery in the paralyzed rats. After 2 days, the length of life of most of this series, the dorsal vagus cells still show an injury almost comparable to that found immediately following the shock.

With these marked changes found in the nerve cells it does not seem strange that numbers of the rats never breathed following the shock and that artificial respiration had to be used in the case of the others. The inactivity for the first 24 hours in those that recovered completely and throughout their lives in the paralyzed rats may be explained on the same basis.

*Rats Subjected to Alternating Circuits at 500, 220 and 110 Volt Potentials and at 1000 and 500 Volt Potentials with the Voltage Not Maintained*

It is scarcely justifiable to discuss the histological picture found in the central nervous systems of each of these groups of rats separately. The relative mortality and paralysis produced at those different potentials have been thoroughly summarized in a previous paper. It is sufficient to point out that the number of rats killed or paralyzed became smaller, as the voltage and the amperage of the circuit were decreased. The shock could be made lethal, however, in every case by increasing the time of current contact.

The changes found in the nervous system were similar to those already described.

Actual burning of the brain occurred only with circuits at 1000 and 500 volts potential. Hemorrhages in the spinal cord were demonstrated in all the rats that were paralyzed following the shock. Hemorrhages were common in the groups in which the potential of 1000 or 500 volts fell considerably, immediately following the initial shock. It must be assumed, therefore, that the initial shock

at high voltage is very important in the development of this lesion. A section from the spinal cord of one of these rats is reproduced in Fig. 1. The conclusion was reached that hemorrhages are seldom responsible for the immediate death of the animal. Except in those cases where a hemorrhage occurs in the fourth ventricle, the respiratory block or paralysis must be due not to hemorrhage but directly to cell damage. This will be clearer when the group of rats shocked with the continuous circuit has been discussed.

As regards the cell changes, they were quite marked in the 500 volt series but decreased markedly in the 220 and 110 volt groups. They could be demonstrated, however, in each group by lengthening the duration of the contact. Recovery of the cells in the rats that survived was more rapid at the low voltages. More of the cells showed the potentialities of recovery. In the groups subjected to the lower voltages, it is probable that death was due, in most cases, to a temporary block in the respiratory center.

#### DISCUSSION

The injuries produced by the alternating circuits were somewhat different from those produced by the continuous. Hemorrhages in the central nervous system were common after contact with alternating circuits; the damage to the nerve cells was more severe after injury with the continuous circuits.

The hemorrhages following the alternating current shocks occurred not only in the nervous system but in other portions of the body. Blood vessels were found to have ruptured in the mucous membranes and autopsy study showed hemorrhages in the striated muscles. Blood escaped into the subarachnoid space, into the ventricles of the brain and the central canal of the spinal cord. Hemorrhages in the fourth ventricle were responsible in some cases for respiratory paralysis. Other characteristic hemorrhages occurred in the substance of the brain and spinal cord. In the latter position they caused in some cases a complete transverse necrosis and were responsible for the paralysis of the posterior portion of the body. As a result of the cord injury, hemorrhages occurred in the wall of the atonic and distended bladder and blood appeared in the urine. In the cord the pressure of the blood produced characteristic histological changes in the ventral horn cells.

The mechanism of these hemorrhages after injury with alternating circuits is not entirely clear. It has been suggested that it is due to the strong tetanic contraction of the back musculature at the time

the circuit is closed. It might on the other hand be attributed to the marked venous congestion that pertains during the shock or the sharp rise in blood pressure immediately following the injury.

Developing hemorrhages in the central nervous system were found in many of the rats that died at once and small hemorrhages in many that appeared to recover completely. If the hemorrhages involved a large area in the brain stem or cord the animal obviously had no hope of complete recovery. These hemorrhages were common with all voltages although they were more frequent with the higher potentials. They were obviously a large factor in the immediate or subsequent death of rats subjected to the alternating circuits.

With the alternating circuits at the higher potentials of 1000 and 500 volts there was injury of the dorsal surface of the cerebral and to a less extent of the cerebellar cortex. These regions were close to the head electrode where the density of the current must have been great. Indeed injuries of the subcutaneous tissue and bone were evident at autopsy. The surface of the cortex was seared and the edge stained an intense blue. Often large cavities were observed in the gray matter of the cortex and, less commonly, scattered throughout the central nervous system. They are supposed to be due to the collection of gases produced by the intense heat of the injury. They were found in rats that appeared normal after the injury. The cavities were surrounded by a deeply staining, condensed area of brain substance. They were not necessarily perivascular. If the rats survived the shock for several days, an abscess occurred on the surface of the cortex in a number of cases. This is interesting in that marked cellular reaction is believed to be retarded following electrical injuries.

The nerve cells showed marked abnormalities particularly with circuits at 500 and 1000 volts. After study of the sections it was believed that an accurate prognosis could be given concerning the death or subsequent recovery of the cells. If the nucleus was shrunken and stained an intense deep color so that chromatin skeins and nucleolus could not be distinguished, the cell was dead. In slightly less severe injuries the nucleolus was swollen and the whole nucleus was shrunken and stained darkly. A cell with a normal clear nucleus has the potentialities of recovery. In many cases the Nissl granules were greatly decreased in number and the outlines of the cells shrunken and irregular.

The cellular injury appeared to be selective so that a few abnormal cells would be found among a group comparatively normal. The Purkinje cells seemed to be most markedly injured and changes in these cells were found even after contact with circuits at 110 and 220 volts. Likewise injured cells were observed in the dorsal nucleus of the vagus, among the somatic motor cells of the cord and in the olives. The primary sensory neurones are resistant to injury.

In the animals that recovered, the cytoplasm of the cells became rather shrunken and stained deeply within a few hours but it was several days before the granules looked normal and distinct. The dead cells became more shrunken and pyknotic and finally disintegrated.

The cellular damage was even more marked in the rats subjected to continuous circuits at 1000 and 500 volts potential. This explains the fact that at 500 volts the continuous circuit was as dangerous as the alternating for the rats; at 1000 volts it was far more dangerous. Almost no hemorrhages were found after the continuous circuit shocks. The changes in the nerve cells have been adequately illustrated in the photomicrographs.

A small number of rats were paralyzed after contact with the continuous circuit but the picture was not the same as with the alternating circuits. There was a tendency to gradual recovery. Incontinence was not as marked a feature and blood was never present in the urine. Moreover there was no sensory loss in the paralyzed area. Microscopic examination showed that these paralyses were due, not to hemorrhage, but to the injury or death of groups of cells in the cord. These cells appeared shrunken and both the nucleus and cytoplasm stained a deep color. An infiltration of the anterior horns was present and phagocytosis of these dead cells observed. Convulsions and priapism occurred in this series and were probably due to the irritation of cells in the nervous system.

The alternating and continuous circuits at 220 and 110 volts potential produced much less injury to the nerve cells. Hemorrhages were common with the alternating circuit. It seems possible that a respiratory block was produced in these groups which might be relieved with adequate, long continued, artificial respiration. It is true, however, that long continued contacts with these circuits greatly increased the damage in the nervous system.

It is believed that definite abnormalities have been demonstrated in the nervous system that account for the death and abnormalities produced in the rats. With the circuits at lower potential these changes are not as marked and it is quite probable that temporary respiratory block was responsible for death. When a large hemorrhage occurred in the brain or cord or where a large number of nerve cells were permanently damaged, recovery could not be expected.

The fact that nerve cell changes may be demonstrated in animals dying at once following the electric shock indicates the explosive suddenness with which these abnormalities are produced. It is almost as if the cell had been blown to pieces by a charge of dynamite. The electric current must react violently upon the electrolytes producing profound dislocations of the nucleus and cytoplasmic material. While most other pathological changes in cells develop slowly this powerful physical agent produces abnormalities that may be demonstrated at once by means of even the rather gross methods of histological study available at the present time.

#### SUMMARY

The alternating and continuous circuits produced different types of lesions in the central nervous system. Hemorrhages were common after alternating current shocks and few hemorrhages were observed in the continuous circuit group. With both types of circuits at 1000 and 500 volts potential, severe abnormalities in the nerve cells were observed. These were more marked in the continuous circuit group. A uniformly staining, shrunken, pyknotic nucleus was taken as a criterion of nerve cell death. The Purkinje cells of the cerebellum were most susceptible to the current. Injured cells were studied in the dorsal nucleus of the vagus, in the somatic motor group, among the primary sensory neurones and in the olives. Changes in the histological structure of the cells in reference to recovery have been discussed.

Injury to the cerebral and cerebellar cortices occurred on the dorsal surface close to the head electrode. Small cavities were produced, particularly in the cerebral cortex, as the result of the circuit contact.

With the continuous and alternating circuits at 110 and 220 volts potential less severe changes were observed in the nerve cells although

hemorrhages were common in the alternating circuit group. It must be assumed in these cases that death was due to respiratory block rather than actual death of the cells.

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#### EXPLANATION OF PLATES

##### PLATE 31

FIG. 1. The rat was subjected to an alternating circuit at 1000 volts for 1 second. A large hemorrhage is present in the posterior columns of the cord. The rat survived the shock and behaved normally until killed 4 days later.

FIG. 2. There is a complete transverse necrosis of the cord following an extensive hemorrhage. The nerve roots appear fairly normal. The posterior portion of the body was paralyzed and there was no sensory response from the paralyzed area. Incontinence followed the shock and large quantities of blood were present in the urine. The animal was inactive and did not eat. The injury followed contact with an alternating circuit at 1000 volts potential for 3 seconds.

FIG. 3. Somatic motor cells of the anterior horn of the spinal cord which have been subjected to pressure from a hemorrhage. The nuclei appear normal except that one occupies an eccentric position. There are few granules in these cells. In the largest cell the granules are more abundant around the nucleus and the peripheral cytoplasm is clear and unstained. This rat was subjected to an alternating circuit at 1000 volts for 1 second.

FIG. 4. This is a somatic motor cell from the anterior gray matter of the spinal cord. The nucleus is normal. The cytoplasm on one side stains deeply and contains many granules; on the other side toward the periphery of the cord the granules are few and the cytoplasm is shrunken and irregular. The rat was shocked with a continuous circuit at 1000 volts for the time required to close and open the switch manually and died at once.

FIG. 5. The nucleus of this primary motor neurone of the cord is normal. Granules are present around the nucleus but the outer cytoplasm is relatively free from them. The rat was injured with a continuous circuit at 1000 volts for  $\frac{1}{2}$  second and died at once.

FIG. 6. This somatic motor cell of the cord must be considered damaged beyond hope of recovery. The nucleus is shrunken and stains a uniform black so that the nucleolus and chromatin skeins cannot be distinguished. It is surrounded by a clear area indicating the normal size of the nucleus. The granules in the cytoplasm look fairly normal. The rat died at once following contact with a continuous circuit at 1000 volts for 1 second.

FIG. 7. The nuclei of these two cells are shrunken and the outlines are irregular. The nucleoli are swollen although they can still be distinguished. They represent an intermediate amount of nuclear damage between Figs. 5 and 6. These anterior horn cells have many granules in the cytoplasm. In the lower cell the cytoplasm is shrunken; the outlines of the cell are irregular and indistinct. The rat was injured by a continuous circuit at 1000 volts for  $\frac{1}{2}$  second and died at once.

FIG. 8. The deep staining cell resembles that in Fig. 6. The other two anterior horn cells have shrunken, vacuolated and irregular cytoplasm with few granules. The lower cell however has a normal nucleus. The rat was subjected to a continuous, 1000 volt circuit and died at once.

FIG. 9. These are greatly shrunken, pyknotic anterior horn cells. The rat was shocked with a 1000 volt continuous circuit. Following the injury the posterior portion of the body was paralyzed. At the end of 6 days the rat was killed. Nucleoli can be observed in the cytoplasm of the two cells on the right. Phagocytic cells have eaten into the cytoplasm of the two lower nerve cells.

#### PLATE 32

FIG. 10. These are normal Purkinje cells. The cytoplasm contains delicate flaky granules.

FIG. 11. Normal Purkinje cells are seen in the upper portion of the photograph. The lower cells are shrunken and pyknotic and no distinction between nucleus



and cytoplasm can be made out. This rat survived a contact with a 1000 volt continuous circuit for 4 days.

FIG. 12. All the Purkinje cells in the photomicrograph are damaged. The nuclei are shrunken and stain a uniform black. The cytoplasm also stains deeply. These cells show the immediate effect of the injury since the rat died at once after a 1 second exposure to a 1000 volt continuous circuit.

FIG. 13. These Purkinje cells show a different type of injury in that the cytoplasm is greatly vacuolated, particularly at the edge next to the granular layer. The rat survived 4 days after contact with a continuous 1000 volt circuit.

FIG. 14. This picture again shows shrunken dead Purkinje cells from the central nervous system of a rat that survived 6 days after contact with a 1000 volt continuous circuit.

FIG. 15. These cells are from the mesencephalic nucleus of the trigeminal and indicate changes in primary sensory neurones. Sensory cells are relatively resistant to electrical injuries. One cell however has a shrunken pyknotic nucleus. The rat died at once after shock from a 1000 volt, continuous circuit for 1 second.

FIG. 16. Two cells of the mesencephalic trigeminal nucleus have injured nuclei. The nuclei are surrounded by clear areas indicating their normal size. The granules in the cytoplasm are not sharply discrete. Again the rat was subjected to a 1000 volt continuous circuit and died at once.

#### PLATE 33

FIG. 17. These are cells from the dorsal nucleus of the vagus. The arrangement of the granules is fairly normal for autonomic cells and the cytoplasm appears to have suffered little damage. The nuclei however stain a uniform dark color and it is thought the cells have been killed. The rat died at once following contact with a continuous, 1000 volt circuit for 1 second.

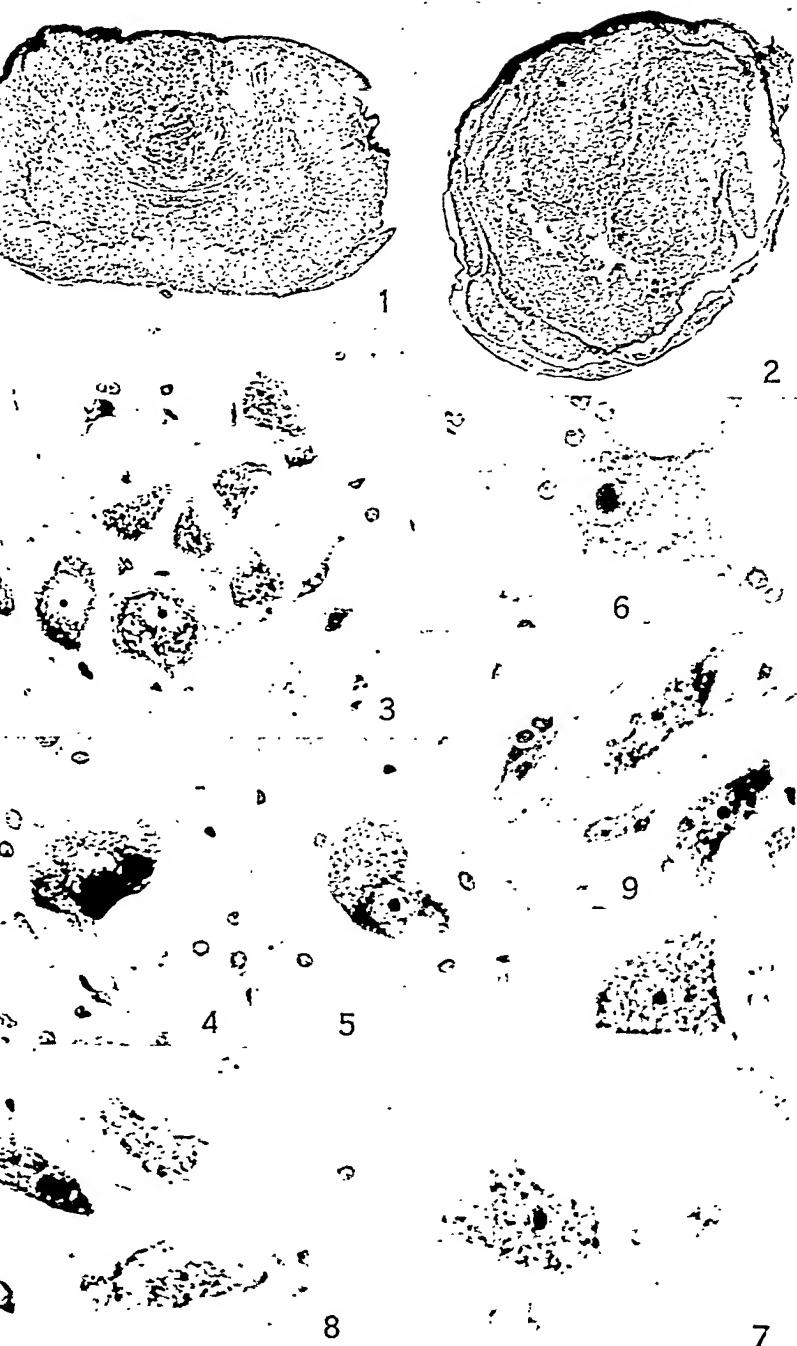
FIG. 18. The dorsal nucleus of the vagus. The upper cells appear normal; the others are damaged. One has a deep staining nucleus. The granules are irregularly arranged and the whole cytoplasm of one cell stains darkly. The rat died at once after a 1000 volt continuous circuit application for 1 second.

FIG. 19. Cells of the superior olive. The rat survived an instantaneous, 1000 volt continuous circuit contact for 4 days. No cell in this group is normal.

FIG. 20. Dorsal nucleus of the vagus. This rat lived 6 days after an instantaneous, 1000 volt continuous circuit injury. The cell in the lower portion of the field is normal. At the upper edge a pyknotic cell is seen. Between these are shadows of two disintegrating cells.

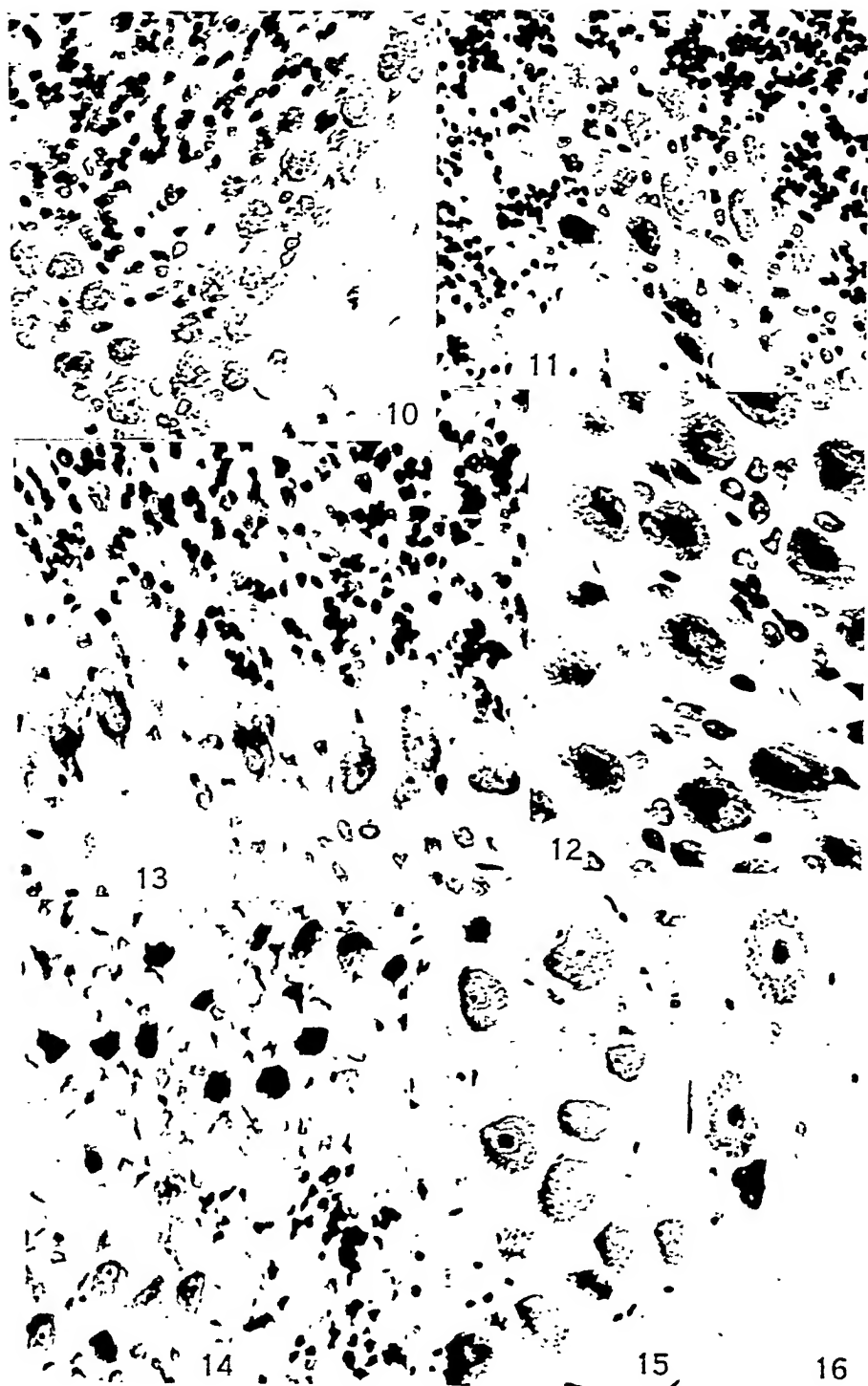
FIG. 21. The distinction between normal and pyknotic cells in the dorsal nucleus of the vagus is clear. The rat lived 4 days after instantaneous contact with a 1000 volt continuous circuit.

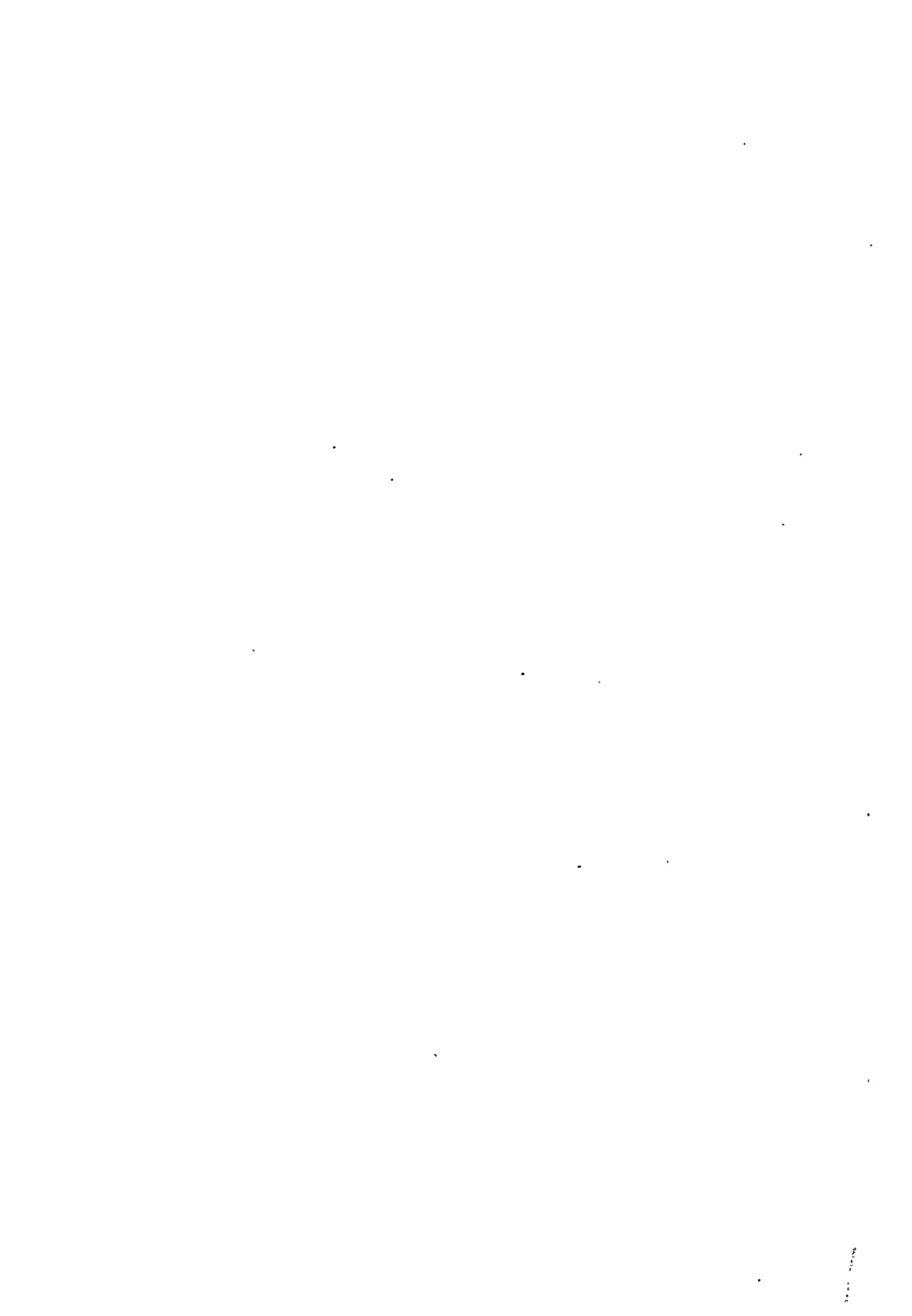
FIG. 22. These cells of the dorsal nucleus of the vagus show severe injury of both the nucleus and the cytoplasm. The rat died at once after contact with a 1000 volt continuous circuit for 1 second.

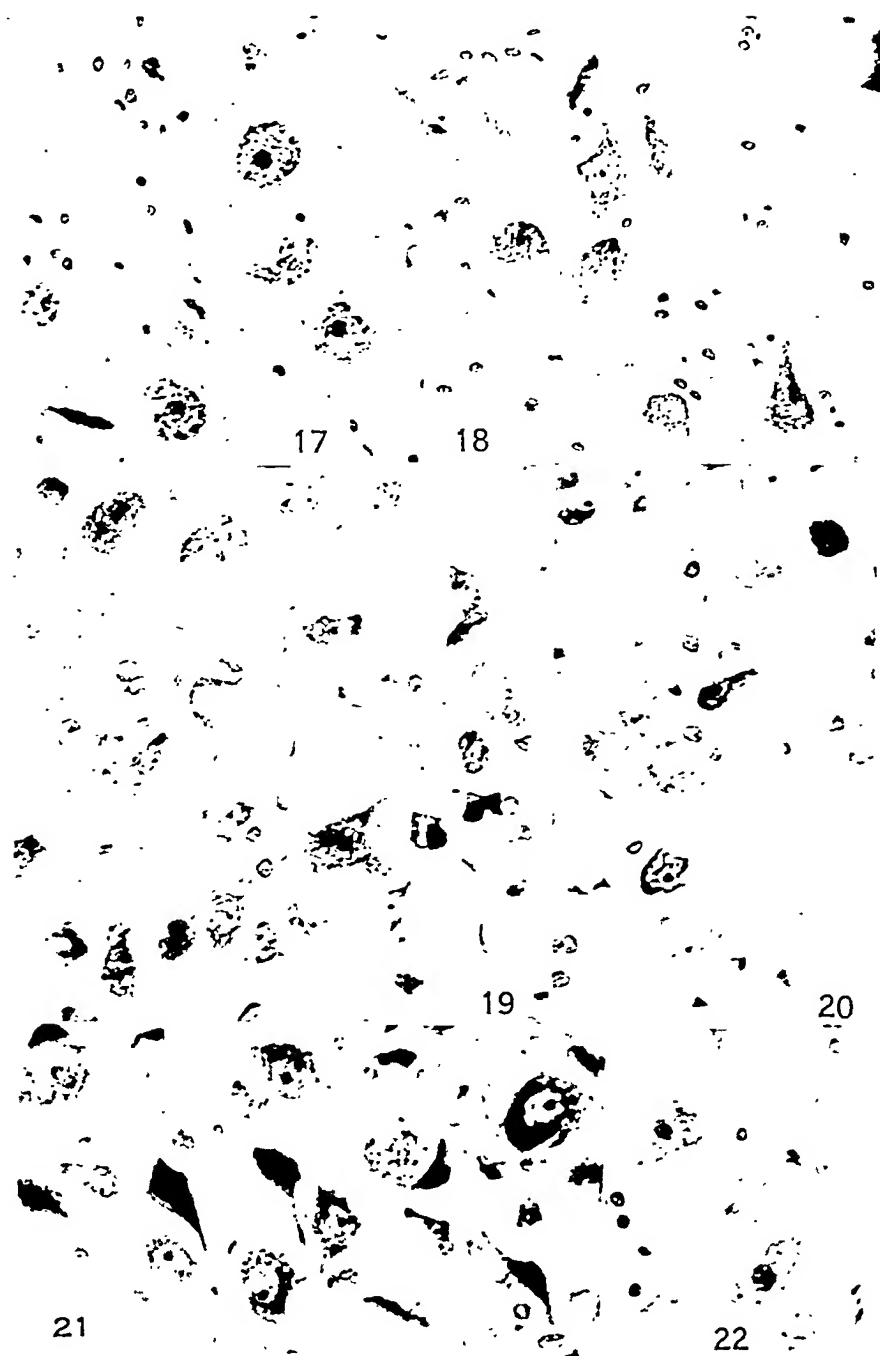


(Langworthy: Electrical injuries to nervous system)









(Langworthy: Electrical injuries to nervous system)



# INFECTIOUS MYXOMATOSIS OF RABBITS

## OBSERVATIONS ON THE PATHOLOGICAL CHANGES INDUCED BY VIRUS MYXOMATOSUM (SANARELLI)

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PLATES 34 TO 37

(Received for publication, March 28, 1930)

In 1927, a preliminary report (1) was made concerning certain features of a disease designated by Sanarelli as infectious myxomatosis of rabbits. The purpose of the present communication is to amplify the previous report and to record some results of subsequent work.

Infectious myxomatosis is a malady indigenous to rabbits of South America and was first described by Sanarelli (2) in 1898. The disease, as he saw it, was characterized by the presence of nodules in the skin in the neighborhood of the eyes, nose, mouth, ears, and genitalia. A conjunctivitis, accompanied by a profuse purulent discharge, resulted from the involvement of the skin around the eyes. The disease ran a rapid course, death of the animals usually occurring within 1 or 2 weeks after infection. Upon palpation the tumors were firm and on section revealed a gelatinous consistency. Histologically they were found to be comprised of gelatinous material, large stellate cells, and blood vessels. The lymph nodes and spleen were enlarged, and, in histological preparations of the latter, nests of the large stellate cells were observed. The presence of the virus was demonstrated in the secretions from the eyes, in the tumors, in the various organs, and in the blood. Besides spreading spontaneously, the malady proved to be transmissible by experimental inoculation. Many kinds of animals, including man, were inoculated. The rabbit, however, was the only susceptible host found. Sanarelli was unable to cultivate the etiological agent on artificial media, nor was he able to see it by means of the microscope. In view of these facts, he concluded that *Virus myxomatosum* is similar in nature to rabic virus.

Since Sanarelli's original communication only 10 papers (see bibliography) dealing with the infectious myxoma of rabbits have appeared. A detailed review of these reports, with the exception of Findlay's (11),



was made by Hobbs (10) in 1928. Therefore, only the results of previous work that are particularly interesting or that have a bearing on the experiments to be presented at this time will be described.

Moses, Hobbs, and Findlay have shown that the myxoma virus is capable of passing through filters impervious to ordinary bacteria. No worker has been able to cultivate the etiological agent on artificial media in the absence of living host cells. The rabbit is the only known susceptible host. Even the wild hares (*Lepus brasiliensis*) of Brazil are resistant to the virus (Moses, 1911).

Splendore, 1909, reported the presence of trachoma-like bodies in the tumor cells. In 1911, Moses stated that he was unable to confirm Splendore's observations concerning these bodies. Aragão, 1911, recorded his observations of certain bodies, *Chlamydozoa myxomac*, within the hypertrophic nuclei of the tumor cells. In 1927, this investigator repudiated his work concerning the nuclear inclusions and stated that the etiological agent is represented by small round granules, *Strongyloplasma myxomac*, situated within the cytoplasm of infected cells. Rivers, 1927, reported that he was able to find large granular acidophilic inclusion bodies in the cytoplasm of epithelial cells covering the myxomatous masses. Hobbs, 1928, confirmed Rivers' observations concerning the presence of these inclusions in the epithelial cells. Lipschütz, 1927, described within the cytoplasm of the swollen tumor cells, the presence of many small bodies which, inasmuch as they seemed to differ from chlamydozoa and strongyloplasms, he designated *Sanarellia cuniculi*. Findlay, 1929, failed to confirm Rivers' observations regarding the presence of cytoplasmic inclusions in epithelial cells. He was able, however, to find Lipschütz's *Sanarellia cuniculi* in the tumor cells.

Although Sanarelli in 1898 described the myxomatous masses in the skin as tumors and found that in infected animals the spleen and lymph nodes were enlarged and that at times an orchitis occurred, certain workers have been unable to confirm his observations. For instance, Aragão, 1927, stated that the myxomatous swellings are not true tumors but remarkable collections of oedema due to an infectious agent. Furthermore, he observed no involvement of the lymph nodes and spleen. Findlay, 1929, reported that "the nodular lesions are thus due, not to an active proliferation of the tissue elements, but simply to the myxomatous changes in the tissues." Moreover, he found "no enlargement of the lymph-glands or spleen." Also, according to him, "the testicles, apart from congestion, were normal even when the scrotum was affected."

From the above brief review of the reports dealing with infectious myxomatosis of rabbits, it is obvious that many conflicting statements and opinions exist. Nevertheless, the disease is so characteristic and so fatal that there is no doubt but that all of the investigators studied the same malady. In fact, many of them were dealing with the same

strain of virus, which originally came from the Oswaldo Cruz Institute in Brazil.

In a preliminary report, 1927, I described for the first time certain changes observed in epidermal cells covering myxomatous masses induced by *Virus myxomatosum*:

Upon microscopic examination the first change noted in the epidermal cells is an increase in their size. Then, small pink, granular areas appear in the cytoplasm. These areas rapidly increase in size and frequently involve most of the cytoplasm. In the center of the acidophilic masses, blue, round or rod-shaped bodies are often seen. . . . The disease process in the epidermal cells progresses until there is complete dissolution of the cells. At this time distinct vesicles appear in the epidermis. . . .

Because of the involvement of the epidermis, a fact that had not been observed previously, I raised the question in my preliminary note as to whether I was dealing with more than one virus. Since that time work on the myxoma has been continued and now it seems advisable to record certain observations that may be of interest.

### *Methods and Materials*

*Virus*.—The myxoma virus was obtained by Dr. C. E. Simon from Dr. A. Moses of the Oswaldo Cruz Institute, Brazil. In May, 1926, Dr. Simon sent the virus to Dr. A. Carrel who gave it to me for study.

*Methods of Inoculation*.—Animals were inoculated epidermally, intradermally, subcutaneously, intravenously, and intranasally. A few animals were allowed to contract the disease through contact with infected rabbits. Except for the tumors that arose at the sites of inoculation, the disease picture was the same following all types of inoculation.

*Cultures*.—Infectious blood and bits of the tumors and different organs were tested for sterility by means of the usual aerobic and anaerobic cultures. No bacterium of etiological significance was encountered. In approximately 50 per cent of the rabbits that died of the myxoma, *P. lepi-septica* was obtained from some organ or tissue. Sufficient animals, however, with sterile tumor masses, blood, and organs were studied to convince one that the changes to be described in this paper were not induced by *P. lepi-septica*. In working with the myxoma, one should endeavor to use rabbits that are not carriers of *P. lepi-septica*.

*Fixation and Staining*.—Tissues used for histological studies were obtained from rabbits sacrificed by the intravenous injection of air. The tissues were fixed in Zenker's (5 per cent acetic acid) fluid and stained with eosin and methylene blue and according to Giemsa's method.

## EXPERIMENTAL

The course of infectious myxomatosis and the histological findings are not materially influenced by the manner in which the disease is contracted. When the virus is rapidly passed in series from rabbit to rabbit, however, it kills the animals so promptly that sufficient time does not elapse for the formation of large and characteristic lesions. Therefore, if large tumors and metastases are desired for study, a virus that has been stored for a long time should be employed.

During a period of 4 years, approximately 100 rabbits have been used for various studies of the myxoma virus. Of these animals, 25 have been carefully autopsied and the tissues have been examined histologically. The results of each autopsy will not be given in detail. The findings in different tissues and organs will be described and the relative frequency of the involvement of these organs and tissues will be noted.

*Skin*

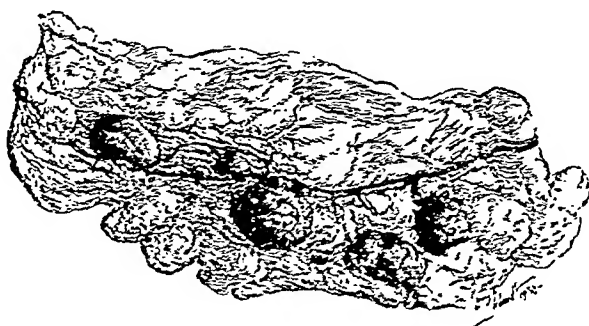
In every susceptible animal the epidermis, corium, and subcutaneous tissues were involved. The appearance of primary and secondary lesions in the skin is illustrated in Figs. 1-7. Such lesions are elevated, round or oval masses, the centers of which may take on a purplish color. Upon palpation the masses are firm and at times have the consistency of cartilage. The affected skin around the genitalia, however, frequently presents the appearance of oedematous tissue (Figs. 6, 7). If the lesions have progressed sufficiently, evidences of vesiculation can be found (Figs. 1, 5). In fact, fluid can be obtained from the vesicles by means of capillary pipettes. If the animals survive long enough, the vesicles are replaced by crusts which cap the tumor nodules (Fig. 5).

On sectioning the tumors one finds them firm, and hard to cut. The epidermis is thickened or shows evidences of vesiculation. The corium and subcutaneous tissues consist of a tough, pinkish, gelatinous material freely supplied with blood vessels. The tumors may be attached to the underlying muscles.

Histological examination reveals the following course of events in the epidermis. The cells increase in number and size (Fig. 9). Then small acidophilic granules appear in the cytoplasm. These rapidly increase in number and eventually replace the major portion of the normal cytoplasm. Among the acidophilic granules round or rod-shaped blue bodies may be seen (Figs. 21, 23). The nuclei become swollen or vacuolated (Fig. 23) and the chromatin is fragmented. Finally the cells undergo dissolution and vesicles appear in the epidermis (Figs. 10, 11).

In the involved corium and subcutaneous tissues an amorphous-looking material is seen in which large stellate or polygonal cells (Figs. 12, 24, 25), many polymor-

phonuclear leucocytes, and multinucleated cells (Figs. 12, 14) are found. The nuclei of the polygonal cells are swollen, and the chromatin is fragmented (Fig. 25). Dividing cells and mitotic figures are not numerous, but they do occur. Many of the so-called tumor cells are strikingly phagocytic. The cytoplasm of these cells contains a large number of granules. Whether some of the granules are similar to those described by Lipschütz and Findlay is not known. It seems, however, that the majority of the bodies represents not virus particles, but ingested material. The myxomatous masses are abundantly supplied with blood vessels, many of which are surrounded by the large polygonal cells (Fig. 12). At times the endothelial cells of the capillaries also seem to be involved (Figs. 12, 13) and are not unlike the large cells seen in other parts of the tumor.



TEXT-FIG. 1. Chain of axillary lymph nodes draining site of primary inoculation of the skin with myxoma virus. The glands were hypertrophic, hemorrhagic and firm. Histological examination of the 4 larger glands revealed the fact that the lymphocytes had been completely replaced by "myxoma" cells. Natural size.

In the epidermis and in the corium affected by the virus it appears that more than a myxomatous metaplasia of the cells already present occurs. Evidences of growth and destruction of cells are found.

### *Lymph Glands*

Examination of the axillary and popliteal lymph glands was a routine procedure of each autopsy. If the animal is inoculated in the skin over the lateral surface of the thorax and upper part of the abdomen, it is usual to find the axillary glands on the same side involved. Glands in the other axilla and in the popliteal spaces, in a great many instances, also show evidences of involvement. A marked degree of hypertrophy is the first change noted; at times the increase in size is tenfold. Then discrete red areas appear on the surface of the glands. Finally the nodes become very firm and hemorrhagic throughout (Text-fig. 1).

Histological examination reveals that the early changes in the lymph glands are characterized by an increase in the lymphatic tissue, *i.e.*, lymphocytes. Then in the lymph sinuses around the periphery of the glands phagocytic cells make their appearance (Fig. 18), many of which are multinucleated. Soon the lymphocytes begin to disappear, and, if sufficient time elapses, the glands become devoid of such cells. At this stage of the disease, the large lymph nodes consist of cells resembling those found in the subcutaneous myxomatous nodules (Fig. 19). These cells are not normal; they have hypertrophic nuclei with fragmented chromatin, and in their cytoplasm different kinds of granules are present, many of which seem to have been ingested. On studying the sections one obtains the impression that these cells arise from the reticulum of the glands. Mitotic figures are not numerous. Nevertheless, they do occur. If the cells that comprise these "metastases" or altered lymph glands arise from the reticulum, their number can be accounted for only upon the assumption that a multiplication of the cells of the reticulum takes place under the stimulus of the virus. In addition to the type of cells just described, many polymorphonuclear leucocytes are seen.

### *Spleen*

The spleen may be enlarged or it may be of normal size. On histological examination nests of large stellate cells are not infrequently observed. In two rabbits, many of the blood vessels in the spleen were surrounded by myxomatous tissue (Figs. 17, 20) composed of cells similar to those described in the "metastases" in the lymph glands. Around each myxomatous nodule was a compact ring of cells, many of which seemed to be spleen cells crowded together by the growth of the myxoma. Other cells in this dense mass doubtless represented the results of host reaction to the infection.

### *Lungs*

As a rule, lesions characteristic of the myxoma were not observed in the lungs. Occasionally a pneumonic process caused by *P. lepi-septica* was encountered. In sections of the lungs from one rabbit, however, numerous myxomatous nodules (Fig. 15) were seen in the vicinity of blood vessels and bronchi. The myxomatous tissue was similar in nature to that observed elsewhere in the body. Moreover, the epithelial cells of the bronchi in close proximity to the myxomas showed evidences of hyperplasia, and, in the cytoplasm of these abnormal cells, changes similar to those seen in epidermal cells were noted.

Inasmuch as the myxoma virus is regularly found in the secretions from the eyes and nose and in the blood, it is difficult to state in what manner the virus that caused the lesions in the lungs obtained entrance.

### *Testicles*

In the majority of the rabbits the scrotal skin is affected, but it is unusual for the testicle to be attacked by the virus. In 4 rabbits, however, myxomatous

changes in the interstitial tissue of the testicle accompanied by a necrosis of the tubules was encountered.

### *Epididymis*

The epididymis (Fig. 8) is involved more frequently than is the testicle. Within the connective tissue, myxomatous masses similar to those in the subcutaneous tissue are found. In one rabbit the epithelial cells lining different parts of the epididymis showed intracellular changes similar to those described in the epidermal cells, *i.e.*, an increase in number and size, and the presence of acidophilic cytoplasmic inclusions (Figs. 16, 22).

### *Tunica Vaginalis*

It is not unusual to find hemorrhagic myxomatous nodules in the tunica vaginalis (Fig. 8).

### *Ovary and Uterus*

Autopsies were performed on 3 female rabbits inoculated with myxoma virus. No lesions were found in the ovaries and uterus. If a large number of infected female rabbits were examined, it is possible that lesions might be encountered in these organs.

### *Pancreas, Liver, Kidney, Adrenals*

No myxomatous nodules were observed in the pancreas, kidney, liver and adrenals.

### *Concerning the Presence of More than One Virus*

The peculiar changes in the epidermis that accompany the myxomatous masses in the underlying tissue induced me to raise the question in my preliminary note (1) whether the observed pathological picture resulted from the combined activity of two viruses. Findlay (11), failing to find the changes in the epidermis described by me, again raised the question as to whether I was dealing with two viruses, one of which is indigenous to American rabbits and is not found in British rabbits. Furthermore, he suggested that one of the agents may be so labile that it is unable to withstand shipment from America to England.

During the last 4 years, I have tried in many ways to demonstrate the presence of more than one virus in the myxoma material and have been unable to do so. Vaccine virus and Virus III have been elimi-

nated as possible contaminants by passage of the myxoma through rabbits immune to these agents. Fowl-pox also is not a contaminant, inasmuch as an emulsion of the myxomatous tissue will produce no disease in fowls. The myxomatous material is innocuous for mice, rats, guinea pigs, and monkeys. Therefore, if two viruses are present in the myxomas, both are specific for the rabbit.

In view of the fact that different viruses are not equally stable, an emulsion of myxomatous tissue in 50 per cent glycerol, and citrated infectious blood were stored on ice for 1 and 2 years respectively in the hope of eliminating in this manner one of the viruses in case two were present. Lesions resulting from inoculations with these materials showed the phenomena in the epidermis that gave rise to the question regarding the presence of two viruses. Therefore, if there are two viruses in the material with which I have been working, then both are extremely resistant to aging and storage or a large number of the rabbits that I have inoculated during the past 4 years have been carriers of the second or contaminating virus. The facts that I have adduced do not conclusively exclude the possibility of the presence of two viruses. Nevertheless, in view of them, such a possibility seems unlikely.

#### DISCUSSION

The fact that maladies induced by viruses are characterized by hyperplasia and necrosis was discussed in a previous paper (12). In the majority of the virus diseases both of these phenomena are observed. In some, however, hyperplasia may play the important rôle, while in others necrosis dominates the picture. Such a lack of balance between the growth and destruction of tissue accounts for the fact that the activity of some viruses gives rise to certain kinds of tumors, *e.g.*, Rous' sarcoma, while the operation of others lead to vesicular lesions, *e.g.*, variola and foot-and-mouth disease.

Many of the viruses are highly species specific. Virus III attacks only rabbits; the salivary-gland virus is active only in the guinea pig; hog cholera is infectious only for swine; each polyhedral disease has its specific caterpillar host; the tumor-forming activity of the Rous sarcoma seems to be limited to chickens; certain mosaic viruses produce pathological changes in one kind of plant. This marked degree

of specificity, however, is not exhibited by all viruses. For instance, vaccinia and rabies may be induced in many kinds of animals.

A close relation exists between the viruses and host cells. This fact is emphasized by the lack of evidence to demonstrate that these active agents are capable of multiplication in the absence of living susceptible host cells, and by the presence in many instances of specific inclusion bodies in the affected cells. The relation between the viruses and cells may be not only close but specific in that certain types of cells alone are directly injured by the disease-inciting agent. For example, in rabies and poliomyelitis, it appears that nerve cells are the susceptible elements. Such cell specificity however, is not observed in vaccinia and herpes febrilis, diseases in which cells of ectodermal and mesodermal origin are involved.

The ease with which virus diseases spread from one host to another varies. Some are contagious, *e.g.*, variola and foot-and-mouth disease; others are only inoculable, *e.g.*, rabies and Rous' sarcoma; while yet others are capable of being transferred only by means of grafts, as is the case with certain infectious chloroses of plants.

The infectious myxoma of rabbits described in the present paper is one of the first maladies placed in the virus group. Moreover, when the available facts are closely examined, it is found to be one of the most interesting and characteristic of the lot. It is highly species specific, contagious, attacks cells of ectodermal and mesodermal origin, and causes hyperplasia and necrosis—the predominance of necrosis in the epidermis leads to vesicles, while the preponderance of hyperplasia in the subcutaneous and other tissues results in tumor-like masses. Moreover, inclusion bodies are found in epithelial cells involved. Finally, the causal agent is filterable, has not been cultivated in the absence of living host cells, and is very resistant to the action of certain chemicals and to long periods of storage.

Infectious myxomatosis, acquired spontaneously or induced experimentally, regularly exhibits foci of metastatic activity of the causal agent. These are always seen in the skin, particularly around the eyes, mouth, nose, and genitalia. The lymph glands draining the site of inoculation are usually involved. Other lymph nodes also may be affected. The epididymis and testicle with its tunic are not infrequently attacked. In an occasional animal, many metastases occur



around the blood vessels of the spleen and rarely multiple metastases are found in the lungs. Inasmuch as the virus is always found in the blood, the word metastases, when used in connection with this disease, does not necessarily mean that multiple lesions arise through the transportation of affected cells from one part of the body to another. The metastases described above probably represent the results of the activity of the virus operating in different parts of the animal.

There is no adequate explanation of the fact that Hobbs and I found changes in the epidermal cells associated with cytoplasmic inclusions, while Findlay in England observed none. Nor is it easy to understand why only a few workers have noted involvement of the lymph glands. In any event, the results of the work reported at the present time indicate that the rabbits in New York over a period of 4 years when infected with *Virus myxomatosum* as a rule exhibited involvement of the lymph glands.

The exact nature of the stellate, polygonal, or myxomatous cells is not known. Lipschütz, (9) speaks of them as histiocytes, while Findlay (11) describes them as "hypertrophied connective-tissue cells." Furthermore, the nature of the inclusions in the epithelial (1) and connective tissue cells (9, 11) is still an open question, as is the case with many of the inclusion bodies in the virus diseases.

I can not agree with the statement of Findlay (11) and Aragão (8) that no active proliferation of tissue elements results from infection with the myxoma virus. The evidence obtained from my work is convincing that both growth and destruction of tissues occur in this disease. Of course the cells evidencing multiplication may be those already present in the tissues. Moreover, a myxomatous change may take place in these cells as a result of the virus activity, but such an occurrence does not alter the fact that an increase in the number of cells also results from the operation of the virus and that this increase plays a part in the formation of the nodules or tumor-like masses. These facts, however, are not proof that the myxomatous masses are true neoplasms similar to cancer in man. In fact, some investigators are not convinced that the exact relation of Rous' sarcoma to true neoplasms has been determined.

In certain respects the myxoma of rabbits resembles the Rous sarcoma of chickens. In others, however, it is quite different. If, as

some believe, the Rous sarcoma appears to be more closely related to true neoplasms than to diseases induced by highly contagious agents, then the myxoma, upon further study, may serve to bridge the gap between the Rous tumor and other virus maladies, and to indicate that, after all, no great difference exists between tumor-forming viruses and those causing vesicular or destructive lesions.

#### SUMMARY

The virus of infectious myxomatosis of rabbits (Sanarelli) induces multiple lesions in the skin, lymph glands, tunica vaginalis, epididymis, testicle, spleen, and lungs.

Growth and destruction of cells in the epidermis overlying the myxomatous masses leads to the formation of vesicles. Cytoplasmic inclusions are found in affected epidermal cells. Occasionally, similar inclusions are seen in other involved epithelial cells. The nature of the inclusions is an open question.

In the myxomatous masses situated in the subcutaneous and other tissues, evidences of alteration and growth of certain cells are observed.

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#### EXPLANATION OF PLATES

##### PLATE 34

FIG. 1. Primary "tumor" surrounded by secondary nodules. Several vesicles cap the primary growth.

FIG. 2. Multiple primary tumors induced by rubbing the virus on the freshly shaved skin. The central portions of some of the nodules reveal a purplish tint.

FIG. 3. Involvement of the eyelids accompanied by a white, purulent discharge.

FIG. 4. Metastatic nodule in lip.

FIG. 5. Metastatic nodule in skin over shoulder. The growth is capped by a vesicle undergoing desiccation.

FIG. 6. Oedema around the vaginal orifice.

FIG. 7. Oedema of prepuce and scrotum.

FIG. 8. Four metastatic nodules, three in the tunic, one in the epididymis.

#### PLATE 35

FIGS. 9, 10, 11. Involvement of epidermis characterized by hyperplasia of the epithelial cells followed by necrosis resulting in vesicles.  $\times 375$ ,  $\times 125$ ,  $\times 125$  respectively.

FIG. 12. Section through a subcutaneous nodule comprised of gelatinous material, large polygonal cells, giant cells, blood vessels, and polymorphonuclear leucocytes.  $\times 250$ .

FIG. 13. Enlargement of capillary, indicated by arrow in Fig. 12, showing involvement of endothelial cells.  $\times 1000$ .

FIG. 14. Higher magnification of giant cell shown in Fig. 12.  $\times 1000$ .

#### PLATE 36

FIG. 15. Metastasis in the lung. The epithelium of the bronchus near the myxomatous tissue is hyperplastic and contains cytoplasmic inclusions.  $\times 150$ .

FIG. 16. Represents the involvement of epithelial cells in the epididymis which is characterized by hyperplasia and cytoplasmic inclusions. Compare with Fig. 22.  $\times 300$ .

FIG. 17. Metastasis around blood vessel in spleen.  $\times 125$ .

FIG. 18. Early lesion in lymph node. Note changes in cells lining the lymph sinus and the presence of large phagocytic cells within the sinus.  $\times 250$ .

FIG. 19. Complete replacement of lymph node by myxomatous tissue.  $\times 250$ .

#### PLATE 37

FIG. 20. Metastasis in spleen. Eosin and methylene blue.  $\times 125$ .

FIG. 21. Acidophilic cytoplasmic inclusions in epidermal cells. Eosin and methylene blue.  $\times 800$ .

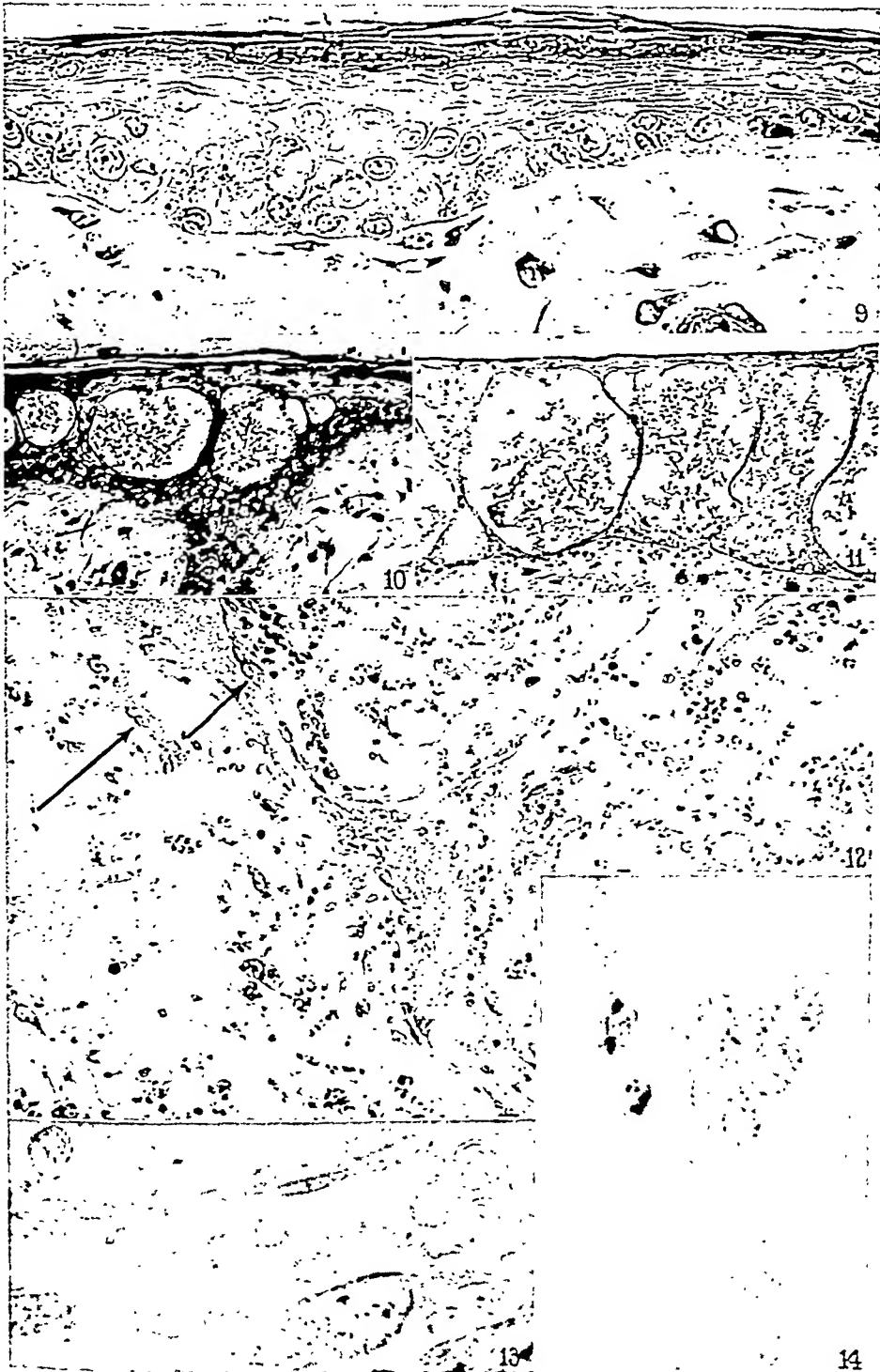
FIG. 22. Acidophilic cytoplasmic inclusions in epithelial cells of epididymis. Compare with Fig. 16. Eosin and methylene blue.  $\times 1000$ .

FIG. 23. Epidermal cell with vacuolated nucleus and granular acidophilic cytoplasmic inclusion in which are situated three blue coccoid bodies. Giemsa.  $\times 1500$ .

FIGS. 24, 25. Myxoma cells. Note fragmentation of chromatin. Giemsa, and eosin and methylene blue respectively.  $\times 1500$ .







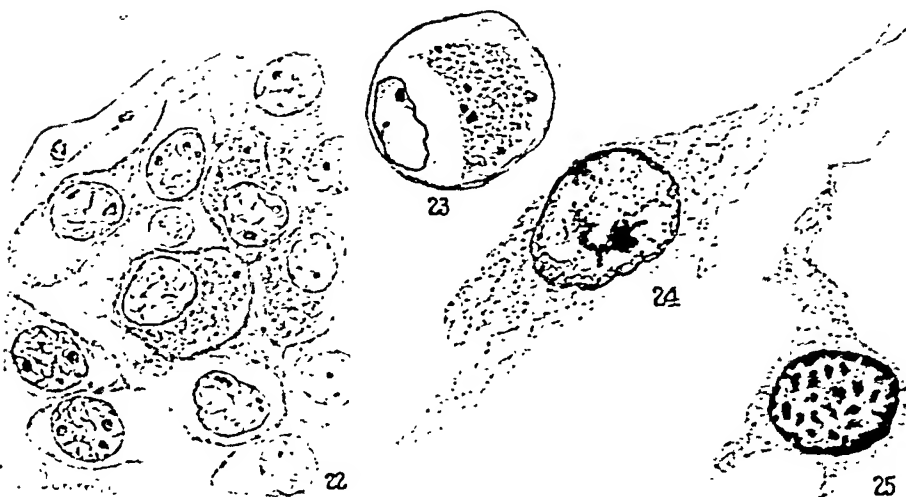
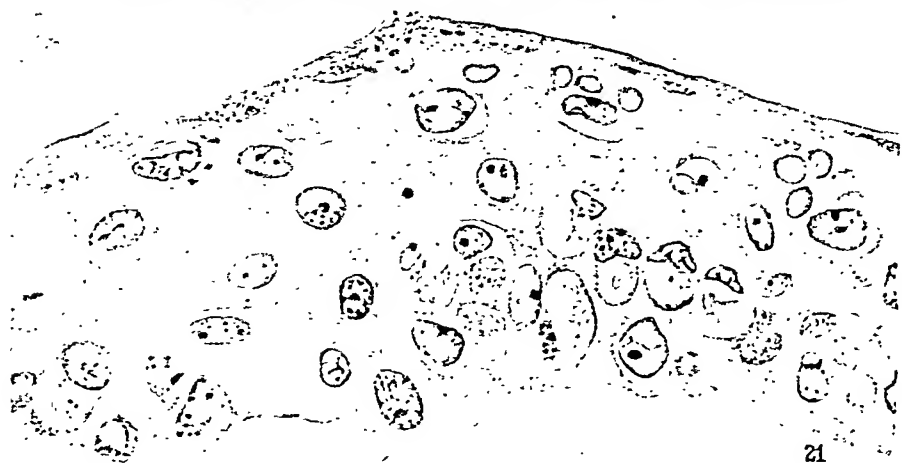
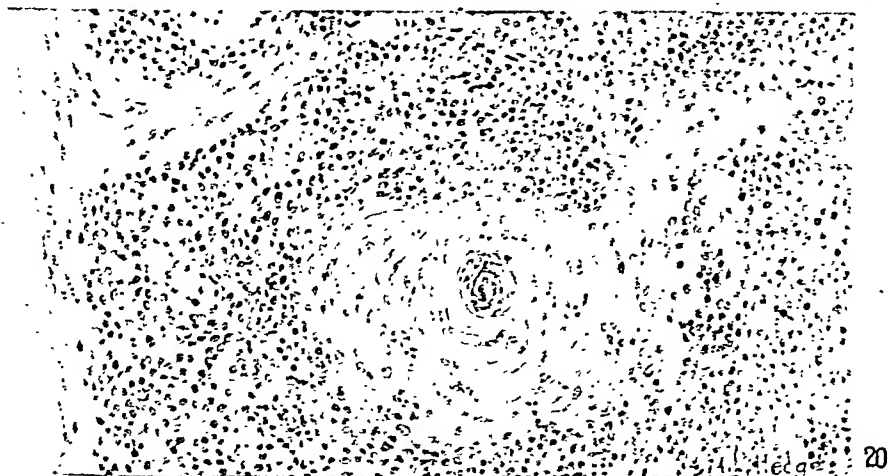
(Rivers. Infectious myxomatosis of rabbits)













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